# PROTEOGLYCANS IN ANIMALS OF MARINE ORIGIN

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## DECLARATION

I declare that this thesis was written by me, and that it represents work performed by me. The publications resulting from the work are quoted in the text and bound at the end of the thesis.

Work which involved other individuals is cited when relevant, and acknowledged.

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## SUMMARY

### Proteoglycans in Animals of Marine Origin

This thesis considers the nature of proteoglycans in cartilaginous fish, mostly elasmobranchs. The main part examines the nature of the jelly found in tubes which form a set of special sense organs, called the Organs of Lorenzini which are served by branches of the facial nerve. These organs have been shown by physiologists to be concerned with electro-perception and enable the animal to detect prey at close range, and to navigate using the earth's magnetic field.

The jelly, which acts as an insulated electroconductor, has been shown in this study to be proteoglycan in nature, the carbohydrate component containing N-acetyl glucosamine, N-acetyl-D-galactosamine, and D-galactose residues. The material is ester-sulphated on carbohydrate residues in all but one of the species examined. The glycosaminoglycan material has been isolated following proteolytic digestion, and some structural studies performed on two species, viz., Squalus acanthias and Galeorhinus galeus. Alkaline degradation of purified material shows that the linkage to protein is an alkali labile glycosidic linkage between threonine and N-acetyl-D-galactosamine.

Whilst the molecule bears some formal relationship to mammalian keratansulphates in its composition, it is none the less significantly different from this material.

Comparative composition studies from a wide range of animal species show that these substances (called now the Lorenzan Sulphates) represent a distinct group of proteoglycan structures which exhibit the characteristic metachromatic staining properties of such polyanions.

A wide variation in the proportion of the two types of hexosamine has been found between the species.

A further secondary investigation into proteoglycans in such animals concerns study of the ageing pattern of this and also of inorganic material found in cartilage tissue. In mammals, ageing changes in the keratan sulphate composition of cartilage demonstrate a marked increase of this with age, but no such change was found to exist in elasmobranch cartilage. The cartilage did show marked ageing changes in its inorganic composition, becoming markedly more calcified as the animal aged, but the proteoglycan composition was found to remain static throughout life, with a constant ratio of keratan sulphate to chondroitin sulphate.
Small amounts of sialic acid were found to be present in elasmobranch cartilage, much less than is present in mammalian cartilage, particularly in old mammalian cartilage.

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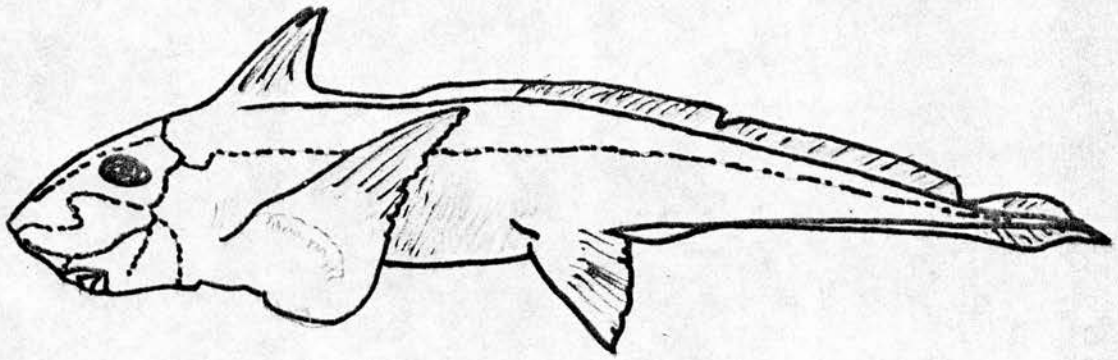

Hydrolagus affinis

Deep water Chimaera. ~100cm long

Plate 3.

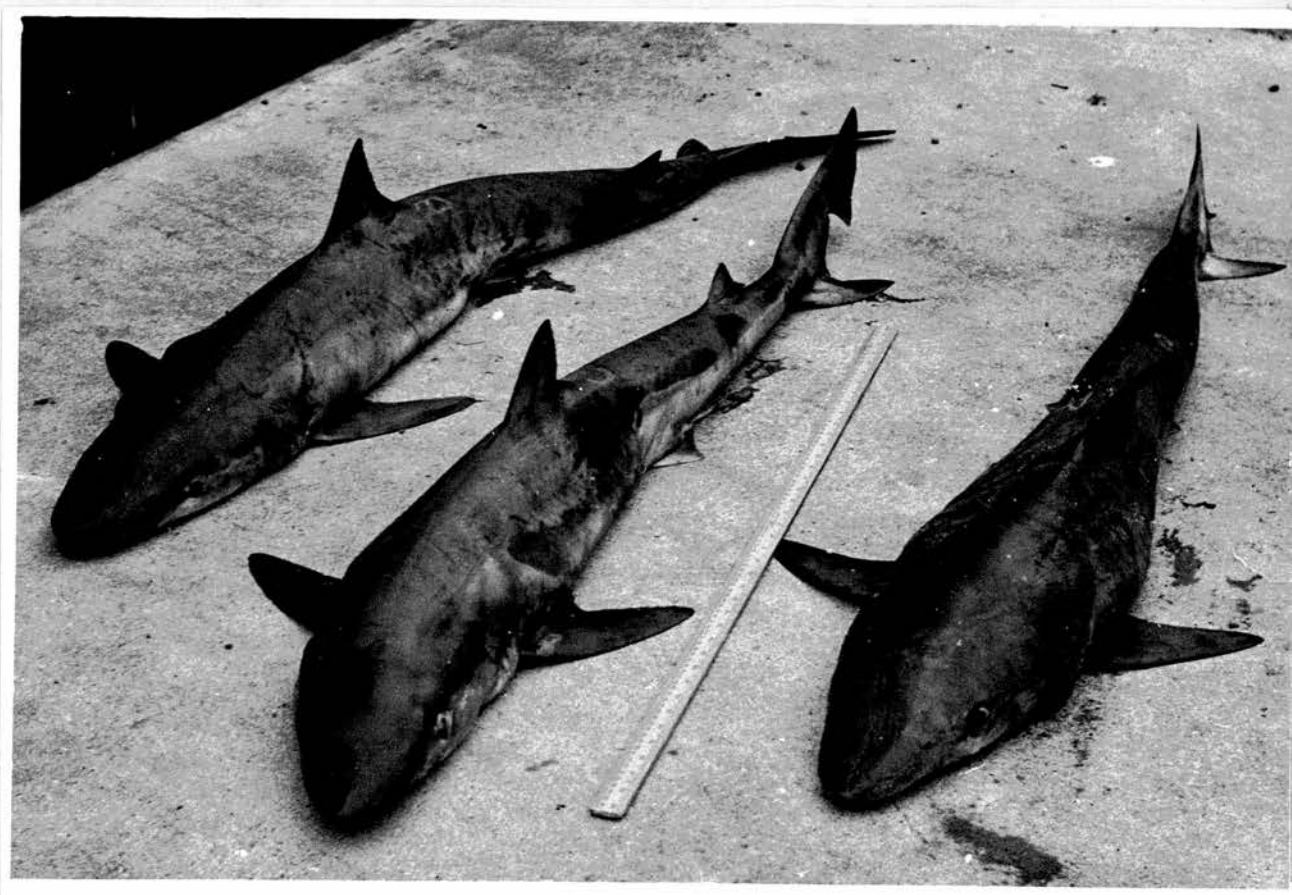

Plate 4. Galeorhinus galeus

Tope

The full length of the rule in the photograph is one meter.

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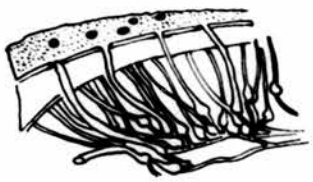

A PORTION OF THE SNOUT IN SECTION SHOWING AMPULLARY TUBES


LORENZINI'S AMPULLA

A. FROM THE SIDE WITH NERVE AND PORTION OF TUBE
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# CHAPTER ONE

## INTRODUCTION

### The Lorenzan Sulphates and Lorenzini's Ampullae. Nature and Structure of the Ampullae.

The organs of Lorenzini are extraordinary specialised sense organs occurring in cartilaginous fish and also in the siluroid, plotosus. The organ consists of an ampulla or bulb-like structure connected to a branch of the facial nerve and which is also connected to the environment of the fish by a tube or canal ending in a pore on the skin surface quite visible to the naked eye. The ampullae and the tubes are filled with a hyaline jelly which varies in "stiffness" from species to species (Plate 5). In the shark-like fish, they are located entirely in the head regions. The tubes thus are fairly short compared with the tubes in the rays where some are found in the snout, but others traverse the entire length of the animal subdermally to end in pores opening in the trailing edge of the wing. Thus in the giant rays, these tubes must be some metres long. The actual size is related to the overall size of the animal and as sharks can be fairly large, the length of the tubes even here can be easily 30 cm and perhaps more. The largest animal dissected in this study was a specimen of the basking shark, Cetorhinus maximus which was some eighteen feet long and had tubes filled with jelly extending from the ampullae deep in the snout to the rear of the head up to 30 cm in length.

Plate 1 shows a dissected head of a modest sized shark, Lamna cornubia, the porbeagle, in which the relations of the ampullae to the nerves, tubes and pores can be easily seen. The size and important position accorded to the organ - it virtually fills the entire snout - provide testimony to its importance to the animal. The function of the organ

is still a matter of some debate, but that it might be a sensitive receptor of electrical fields was suggested by Dijkgraaf and Kalmijn (1962) who noted that blindfolded sharks turned away from a rusty steel wire. More recently, Kalmijn (1971) has published an elegant physiological study in which he found that sharks and rays can respond to a voltage gradient of 0.1 uV/cm. Denervation of the ampullary system abolished the responses. The ampullae are not sensitive to direct current fields, but the animal could appreciate their strength and direction by merely turning its head. Kalmijn (1966, 1971) showed that the animals were able to detect the muscle potentials of live plaice buried in the sand, although only at fairly close quarters, and thus the organ may be used to detect live prey even when buried. The bioelectric fields produced by animals in sea water fall off rapidly with distance and the effect is thus noticed only over short distances. It is clearly speculative to consider the importance of this to the animal in the field. Faraday (1832) observed that all water movements crossing the lines of the earth's magnetic field will give rise to potential differences by electromagnetic induction. Thus ocean currents such as the Gulf Stream and tidal currents which occur close to continents produce electric fields. These are of sufficient magnitude to be well above the threshold of perception of sharks and rays. Akoev et al. (1976) have studied the response of individual ampullary nerve fibres to both electrical and magnetic stimulation. Increase and decrease in magnetic flux elicited a response from a single electroreceptor fibre. The character of the response to magnetic field changes depended on the direction of the field and on the direction of the ampullary canal. The longer ampullary canals were more sensitive to magnetic stimulation than the shorter ones. The authors interpret their findings by postulating that the responses derive from e.m.f.'s produced in the

ampullary canals by changing magnetic fluxes crossing the fish's body. The pores of the canals facing forward and those facing caudally (the animals used were rays, notably Raja clavata) responded to magnetic fields of opposite polarity i.e. North or South poles. This is explained by invoking electrical coupling between receptors causing induced current flow along the canals of opposite direction. The authors conclude that the animals can orientate themselves in the geomagnetic field, some receptors only firing in a certain orientation to magnetic north, and thus acting as a compass. In this study, we are concerned with the nature of the conductor material in the canals. It must be open to the sea for electrical coupling between separate ampullae to take place, and must connect at the ampullary end to the nerve fibre. The jelly-like material is probably continuously synthesised by the cells lining the ampullary canals, as it is soluble in water with some agitation, and must be steadily removed by the motion of the fish through the water. The pore openings in these fish are sizeable and increase with the size of the animal. In the animal mainly studied in this context, the spur-dog-fish Squalus acanthias, animals around 60 cm in length had round pore openings of about 1 mm diameter, and an individual animal might have around one hundred such systems. In the large shark examined, Cetorhinus maximus, the pores were elliptical as were the canals themselves, and the jelly much stiffer in consistency. The least stiff jelly, and most readily solubilised, was found in the smaller rays.

Previous Knowledge of the Chemical Nature of the Jelly from Ampullary Canals

Jensen (1956) made preliminary studies on the nature of the jelly. He observed that solutions were metachromatic with toluidine blue (without giving the conditions) and concluded that the undialysable material was acid mucopolysaccharide in nature.

Jensen made a preparation of material which he subjected to treatment with hyaluronidases, both of bacterial and testicular origin. By measuring the changes in viscosity produced in the solution, he concluded that the material was a mixture of hyaluronic acid and chondroitin sulphate. As this seemed scant evidence on which to base such conclusions, the first part of this study began with more detailed biochemical analysis of the jelly material.

The structural studies have been conducted at a more detailed level than the simple composition of the material, and have concerned the nature of the linkage of the carbohydrate to protein. Comparative composition studies on material from a wide range of elasmobranchs, and from two holocephalic fish have been made, and the proteoglycans from a number of animal species have been isolated and studied. (How, Jones and Doyle, 1969).

In this thesis, the material studied in some depth is from (a) Squalus acanthias, the spur dog-fish, and from the small shark, Galeorhinus galeus, the tope. (Plate 4)

The Linkage of Carbohydrate to Protein

The nature of the linkage of carbohydrate to protein in proteoglycans has been much studied in recent years, and important developments in our understanding of such structures, mainly in mammalian proteoglycans, have been achieved. The importance of the protein component in the formation and structure of these complex polyanions has been clarified. As with the more general group of glycoproteins, proteoglycan structures cannot be formed unless the correct foundation protein is first formed on the ribosomes. Thus the carbohydrate section is a post-translational modification of the fundamental protein structure, and is genetically coded for only indirectly in the sense that the appropriate glycosylating enzymes

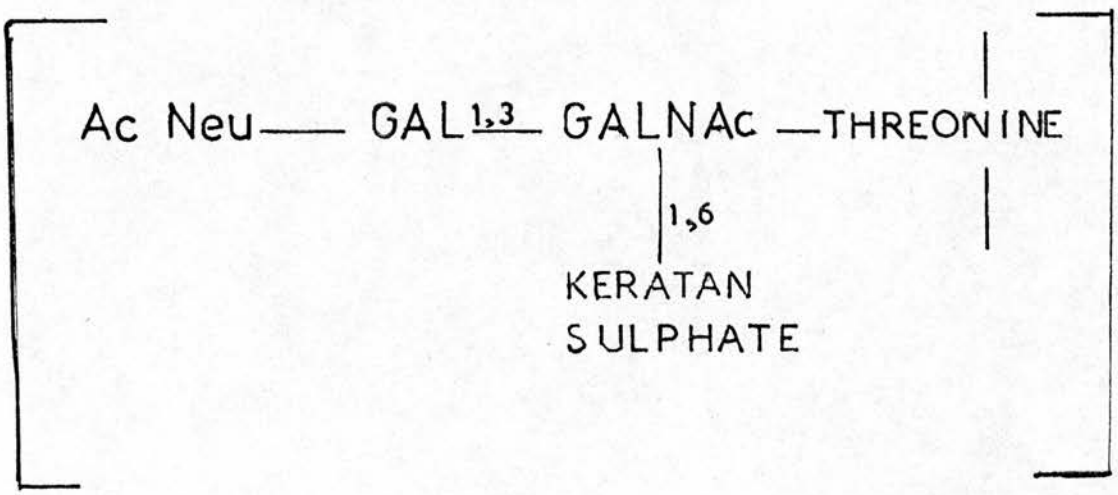
are gene controlled. Thus the modern view of the structure of these substances is of a protein backbone with a number of carbohydrate (glycosaminoglycan) chains radiating from this at centres of glycosylation. In the proteoglycans, these polysaccharide structures are usually unbranched, but a limited amount of branching has been described for the keratan sulphate chain (Hopwood and Robinson,1974) accommodating the sialic acid residues at the branch termini. The protein component of at least some proteoglycans plays a further role in the higher order (aggregation) structures of those macromolecules. This protein component is involved in aggregation with specific proteins and proteoglycans in cartilage to form very large complexes (Gregory 1973; Heinegard & Hascall 1974). As the glycosaminoglycan structures are highly anionically charged, they are likely to exist in an extended state in extracellular spaces due to repulsive forces, giving rise to the "flue-brush" model for their structure.

The nature of the linkage between the carbohydrate and protein in one of these systems was first hinted at by Muir (1958) who showed that serine was involved. After papain digestion, serine was the only amino acid whose concentration was not greatly reduced with respect to the carbohydrate component. The alkali-lability of the bond between chondroitin sulphate and the protein to which it is attached was considered by Muir to mean that an ester bond or something similar was involved. In chondroitin - 4 - sulphate, the linkage to serine was further demonstrated by Gregory et al. (1964) who degraded the complex enzymatically and showed that serine was the predominant amino acid found in isolated fragments, accounting for 96% of the amino acids found in one fragment.

Anderson, Hoffman and Meyer (1965) showed that alkali catalysed the $\beta$ - carbonyl elimination of an alkoxide from an O - substituted serine, thus releasing the chondroitin sulphate chain from the amino acids. After alkali treatment, the serine was found to be converted to a dehydroalanyl residue which was identified as an increase in alanine after reduction. Lindahl and Roden (1965) showed that chondroitin sulphate degraded enzymatically followed by mild acid hydrolysis yielded a carbohydrate-serine compound, 4 - O - $\beta$ - D - galactopyranosyl - O - $\beta$ - D - - Xylopyranosyl - L - serine, establishing the linkage as a glycosidic one between xylose and the hydroxyl group of serine.

In heparin, Lindahl and Roden (1965 a ) isolated a galactosylxylosylserine suggesting a linkage structure for this polymer similar to that found in chondroitin - 4 - sulphate. In keratan sulphate from cornea, (Seno et al.,1965) the majority of carbohydrate groups proved stable to alkali and appeared to be linked via N - glycosyl groups, whereas in skeletal keratosulphate II (author's nomenclature) the linkages were via O - glycosidic bonds to both serine and threonine which are eliminated by mild alkaline treatment. Thus cartilage keratosulphate of mammalian origin differs fundamentally from mammalian keratosulphate found in corneal tissue. A further observation by these authors is that skeletal keratosulphate and corneal keratosulphate contain variable concentrations of methyl pentose (fucose) and sialic acid, both in terminal positions attached to the carbohydrate chains. Bray et al (1967) investigated the linkage in keratan sulphate (keratan sulphate is the more recent term used for keratosulphate, but the usage is not yet universal) and showed that alkali treatment of rib cartilage keratan sulphate gave a Morgan-Elson chromogen that remained attached to the keratan sulphate

chain, and suggested that in keratan sulphate, N-Acetyl galactosamine is linked O-glycosidically to a serine or threonine residue in the protein. They proposed that the chromogen is produced by $\beta$-elimination of a substituent attached to position 3 of the N-Acetylgalactosamine and that the keratan sulphate repeating carbohydrate chain is linked to position 6 of the same N-Acetylgalactosamine residue. Meyer (1970) showed that in cartilage keratan sulphate, after alkaline elimination the peptide chain still remained attached to the carbohydrate chain despite the conversion of the N-Acetylgalactosaminyl group into a direct Ehrlich chromogen. Because of this, the existence in the cartilage keratan sulphate of a second type of alkali-stable bond was postulated. Hopwood and Robinson (1974 )studied the degradation of cartilage keratan sulphate, and showed that the linkage N-Acetyl Galactosamine was further substituted on position 3 :-




In a further study of the composition of skeletal keratan sulphate, Hopwood and Robinson (1974a) showed that per keratan sulphate chain 1 residue of mannose, 3 residues of N-acetyl neuraminic acid, 1 residue of N-acetyl galactosamine (lost on alkali treatment), and up to one residue of fucose were found. Confirmation of two different types of linkage between skeletal keratan sulphate and protein was found.

The alkali-stable link appears to involve glutamic acid, perhaps in amide linkage to glucosamine.

Hopwood and Robinson (1974a) point out that the presence of fucose, mannose, sialic acid, of the O-glycosidic link to protein through N-acetyl galactosamine, and the branched nature of keratan sulphate is similar to the situation occurring in sulphated glycoproteins, e.g. chick allantoic membrane glycoprotein as described by How and Higginbotham (1970) and (1970a).

The material found by the author in this thesis will be seen to have at least some of these features, and might be considered to have affinities with this broad group of substances.

The second part of this thesis considers proteoglycan material from one of the same animals, the dog-fish, Squalus acanthias, but from a different tissue, namely cartilage. Many detailed structural studies have been made on the proteoglycans of shark cartilage notably the chondroitin sulphates and the keratosulphates (Seno et al 1964). These authors examined keratosulphate fractions in the blue shark, Prionace glauca, and compared them withfractions prepared from mammalian cornea and cartilage. They found in the shark keratosulphate, glucosamine:galactosamine ratios of 12:1 which is similar to the situation in old human rib cartilage but much different from corneal cartilage where glucosamine galactosamine ratios of 50:1 are found in the purified keratosulphate fractions.

In the present study, an investigation was conducted into a comparison of differences between elasmobranch cartilage and mammalian cartilage from the point of view of the age changes in their composition. Shetlar and Masters (1955) first observed ageing changes in mucopolysaccharide content of human costal cartilage. Both uronic acid and hexosamine concentrations were found to decrease with age, but not in the same way, implying that not all of the changes were in uronic acid containing polysaccharide. In 1958, Stidworthy et al demonstrated that galactosamine concentrations in human cartilage decreased with age at the same rate as uronic acid concentrations. Kaplan and Meyer (1959) also using human costal cartilage showed that if the ratio of keratosulphate to total mucopolysaccharide is plotted against age, a linear correlation is found. This means that the keratosulphate fraction increases with age even though the total mucopolysaccharide concentration in the ground substance is decreasing with age.

Anderson and Odell (1960) studied changes in rat cartilage with age and radiation and noticed a fall in chondroitin sulphate levels, particularly marked during the maturation period and they considered the changes represent a maturation rather than a senescence. In the human nucleus pulposus, Hallen (1958) noted an increase in the ratio of glucosamine to galactosamine from about 0·5 at 15 years of age to 1·5 at 90 years. He interpreted this to mean a rise in the ratio of keratosulphate/chondroitin sulphate, as the amino sugar characteristic of keratosulphate is glucosamine whereas chondroitin sulphate (either A or B) contains galactosamine. These proteoglycans are now known to be more complex than this since Partridge and Elsden (1961) showed that chondromucoprotein of cartilage contained variable

amounts of glucosamine and galactose which might be an integral part of a complex macromolecule. Tsiganos and Muir (1967) isolated an antigenically distinct protein consisting of a single antigen containing keratan sulphate linked to material analytically similar to chondroitin sulphate from pig laryngeal cartilage. This appeared as direct evidence for what was becoming suspected by many workers, i.e., that the proteoglycan existed as a hybrid species with two different types of repeating disaccharide glycosaminoglycan chain. These are, chondroitin sulphate which has D-glucuronic acid and N-acetyl D-galactosamine as its component sugar residues, and keratan sulphate, which has D-galactose and N-acetyl D-glucosamine as its repeating sugar residues. Tsiganos and Muir (1967) showed by immunological methods, that the protein to which keratan sulphate and some chondroitin sulphate were attached was different from the protein to which most of the chondroitin sulphate is bound. Thus, as a mammal ages, the expression of the genetic information must change to increasing relative production of the hybrid type of protein, wherein glycosylation of separate chain types proceeds on the same protein, as compared with production of the protein which is recognised only by those glycosylating systems producing but one type of gycosaminoglycan chain. A complex structure for the hybrid in which the chondroitin sulphate is linked via xylose to a serine residue on the protein backbone, and the keratan sulphate is linked via N-acetyl galactosamine to a threonine residue on the same protein, emerges from the work of various authors, (Meyer et al. 1964; Bray et al. 1967.) A further complication appears to be the existence of an alkali-stable bond between glutamic acid (or glutamine), and keratan sulphate chains.

The chain is glycosidically linked to serine and is, in consequence, alkali-labile and another site on the same chain appears linked to

glutamic acid in a manner which renders it alkali-stable.

These studies on the nature of keratosulphate have been conducted largely on mammalian cartilage. As the animal ages, the amount and proportion of the keratan sulphate hybrid increases relative to chondroitin sulphate. As the situation in elasmobranch cartilage had not been investigated in this regard, it was decided to take advantage of the large numbers of Squalus acanthias being used for the study of the material from Lorenzini glands. From this material, an investigation was conducted into whether these well documented age-changes in mammals obtain in a lower vertebrate.

# CHAPTER TWO

## EXPERIMENTAL PROCEDURE

### Collection of Material from Organs of Lorenzini.

The fish from which the material was collected were trawled in the Clyde Sea Area and the samples were collected at the Marine Station, Millport on the same day. A few of the samples, including *Chimaera monstrosa*, *Torpedo nobiliana* and *Hydrolagus affinis*, were trawled or line caught elsewhere, by the Torry Marine Laboratory, Aberdeen, or in the Bay of Biscay by the Marine Biological Station, Plymouth. The heads of these animals were sent to me frozen in ice and were thawed out on arrival and immediately dissected for material.

In the case of the smaller animals, e.g. *Squalus acanthias*, material was collected without dissection by pressure with a stiff spatula in the region of the pores. About a gram of jelly could readily be collected in this way, and although this probably represented a small fraction of the total material, plentiful supplies of such animals usually made dissection unnecessary.

The material from the rays was collected in the same way except that the lines were followed on the skin to the pores on the leading and trailing surfaces of the wings. More care had to be used in collecting material from rays, as these animals secrete a copious supply of glycoprotein as skin mucus which could contaminate the collection of Lorenzini jelly.

In the larger animals, particularly the large sharks such as *Cetorhinus maximus*, the basking shark, material was collected by

dissection. Plate 1 shows a dissected head of Lamna cornubia caught off the Hebrides, and illustrates the main points of the anatomy of this intriguing organ.

After collection, the material was stored at -20°C. Some of the material used immediately was treated as wet jelly for analytical purposes, and some was dissolved in water with stirring and dialysed against running water prior to freeze-drying. This freeze dried material was used in most of the structural investigations. Recovery of material after dialysis and freeze drying is shown in Table 1.

Table 1 Recovery of dialysed material

| | Wet wt (g) | Dry wt (g) |
|---|---|---|
| Squalus acanthias | 10 g | 0.51 g |
| Galeorhinus galeus | 10 g | 0.46 g |

Preparation of "Protein-Free" Material

The above material was subjected in batches of 1 g to proteolytic digestion, as the nitrogen analysis on both the dialysed freeze-dried material and the original jelly indicated the possible presence of protein. The results of nitrogen analysis of the original jelly and of the dialysed freeze-dried material in Table 2. (% w/w).

Table 2 Nitrogen content of Lorenzini jellies

| | Wet Jelly | Dialysed dried |
|---|---|---|
| Squalus acanthias | 0.76 % | 5.8 % |
| Galeorhinus galeus | 0.62 % | 5.5 % |

Some of the nitrogen of the jelly in the original wet state is accounted for by the presence of urea which is present in Raja clavata jelly to the extent of 74·8 mM/Kg water (Murray & Potts 1961).

Two procedures were used for digestion of the material (a) pronase digestion and (b) papain digestion.

Pronase Digestion

Dialysed, freeze-dried material from both the main species investigated was incubated with Pronase-B (Calbiochem.Ltd.).

Batches of dried Lorenzini jelly were dissolved with shaking (1g) in 0·05 I Tris buffer (200 ml) pH 7·7 at 37°C. Calcium ions were added ( $CaCl_2$ ) to a final concentration of 5·0 mM. 100 mg pronase was added with shaking and the mixture incubated at 37°C for 36 h. A few drops of toluene were added as a bacteriostatic agent. After incubation, the mixture was filtered and dialysed against running water for 24 hours. This material was concentrated by freeze-drying and subjected to preliminary purification.

Papain Digestion

10 g batches of dried jelly (or on occasions, 100 g batches of fresh jelly) were digested with papain (BDH) in the presence of activators as described by Scott (1960). To the 10 g batches, 250 mg crude papain were added, dissolved in 300 ml 0·1M phosphate buffer, pH 6·5. The mixture was adjusted to 5 mM with respect to ethylene-diamine-tetra-acetic acid and to cysteine. The final solution was made molar in terms of NaCl. The digest was incubated for 24 hours at 65°C in the presence of toluene as a bacteriostatic agent.

Following digestion (by either enzyme) the material was dialysed overnight against running tap-water and finally against three changes of distilled water at 5°C with a volume ratio 50:1 (outside: inside). The solution was centrifuged (3000g : 20mins) decanted and concentrated to approx. 20% of its original volume in a rotary evaporator at 50°C. The solution was again centrifuged (12,000 g, 5°C for 20 mins.) and then decanted. At this stage, six volumes of cold ethanol were added and the material allowed to precipitate at 5°C for 24 hours. The material was collected by centrifugation and washed with ethanol:- yield from 10 g jelly, 290 mg crude powder.

Purification Trials of the Proteolytic Digests

The first technique employed was fractional precipitation of the calcium salts which had been used by Meyer et al (1953) in separating keratosulphates and chondroitin sulphates. As indicated in the results chapter ( 3 ), preliminary analysis of the material from Squalus acanthias had indicated that a mixture of these substances might comprise the carbohydrate components. The ethanol precipitate (30 mg) was eluted with water (50 ml in 2 ml fractions) from a column of Zeokarb 225 $H^+$ form (200-400 mesh) cation exchange resin, 20 cm x 2 cm. The effluent was analysed for neutral carbohydrate by the anthrone method, and the anthrone positive fractions pooled and concentrated to a volume of 4·0 ml on a rotary evaporator. This solution was adjusted to an acetic acid concentration of 0·5 M, and calcium acetate added to a final concentration of 5%. Ethanol was added to produce a series of concentrations ranging between 25 and 80% ethanol, and the material was left at 4°C for 24 hours. After each

individual addition of ethanol, any precipitate formed was collected by centrifugation and retained for analysis.

Trial Purifications

The digest material was subjected initially to fractionation by gel filtration on Sephadex G 50. The main contaminant was considered to be the papain itself, together with peptide material from the proteolysis. Columns of 30 cm x 2.5 cm were equilibrated with water, and 5 ml portions of the crude undialysed digest were applied and eluted with water. Fractions ( 4 ml) were collected, and were analysed for neutral carbohydrate (anthrone analysis) and for protein by the method of Lowry et al (1951), ( Fig 19). All fractions pooled and collected were dialysed and freeze dried.

Trial separation of part-purified digest on Ion-exchange columns.

The neutral carbohydrate containing material from the Sephadex G 100 fractionation of Squalus acanthias jelly was examined for polydispersity on the basis of charge on a DEAE-Sephadex A50 column, 40 x 2.5 cm. The G 100 purified material ( 100 mg) was applied in 0.1M Tris buffer, pH 8.0, in which the column was also equilibrated. The column was eluted with the buffer and then step-wise with buffer containing increasing salt concentrations of sodium chloride.

40 x 2.5 cm DEAE-Sephadex A50 columns: Elution Protocol

| | | | |
|---|---|---|---|
| 0.25 | M | NaCl | 100 ml |
| 0.5 | M | NaCl | 100 ml |
| 0.75 | M | NaCl | 200 ml |
| 1.0 | M | NaCl | 100 ml |
| 1.5 | M | NaCl | 200 ml |

Preparative chromatographic purification of the proteolytic digests based upon these analytical runs.

Approximately 150 mg aliquots of the crude papain digest were purified further in batches. The Sephadex G100 columns, 42 x 5cm, were equilibrated with 1% NaCl and eluted with this salt solution. The effluent was collected in 10 ml fractions, 0.1 ml aliquots were taken for carbohydrate analysis using the phenol/sulphuric acid assay, and for protein directly using absorbance at 280 nm. The carbohydrate containing peaks were pooled, dialysed and freeze-dried. All chromatographic runs were performed in the cold room at 5°C.

In the case of the Tope, preliminary analysis suggested that Sephadex G150 gave rather better separations, and this was used in the subsequent runs using a column of dimensions 30 x 2.5cm. Salt solution (1%) was used as the eluting agent, and 5 ml fractions were collected and aliquots analysed for neutral carbohydrate and protein, (Fig 34).

Fractionation on DEAE Sephadex of Papain Digested Jelly Previously Purified on Sephadex G100.

Samples, (850mg) of material from Squalus acanthias ( S.a.Lj-G100 ) were applied to DEAE Sephadex columns, 70 x 6 cm. which were equilibrated with 0.1M Tris-HCl buffer, pH 8.0. The sample was dissolved in10 ml of the buffer, and the column was eluted with solutions of the buffer with stepwise increases in its salt concentration. (Fig.22) The following protocol of elution was used:

| | | | | |
|---|---|---|---|---|
| 1) | pH 8.0 | Buffer | | 21. |
| 2) | " | " | 0.2M in NaCl | 31. |
| 3) | " | " | 0.4M in NaCl | 21. |
| 4) | " | " | 0.6M in NaCl | 21. |
| 5) | " | " | 1.2M in NaCl | 21. |

The eluate was collected in 25.0 ml fractions, and aliquots of these were analysed for neutral carbohydrate using the Phenol/$H_2SO_4$ method, and for protein with the Coomassie Brilliant Blue G250 dye.

Further Fractionation of the main Fraction from the DEAE Sephadex Column on Ultrogel AcA34 Agarose-Polyacrylamide Gel.

The main fraction (F2) was subjected to gel filtration on a column of Ultrogel AcA34 (L.K.B.). The sample was dissolved in 5.0 ml of the buffer used in the equilibration and elution of the column i.e. 0.1M tris/ HCl, 0.2 M in NaCl, 1 mM with respect to EDTA, and 0.01% sodium azide as a preservative, pH 7.5. The column was 85 x 2.6 cm., and fractions of 5.0 ml volume were collected and analysed for carbohydrate by the Phenol/$H_2SO_4$ method (Fig 25).

The eluate was divided into two fractions as indicated in Fig. 25. These fractions were dialysed and freeze-dried independently, and retained for analysis. Systematically, they are labelled,respectively:

Lj- S.a./G100/ DEAE -F2 / A34-F1 and
Lj- S.a./G100/ DEAE- F2 / A34-F2

Calibration of Ultrogel AcA34 columns was performed using proteins of known molecular weight, viz. bovine serum albumin, thyroglobulin, and myoglobin. (Fig. 24).

Purification and Fractionation of the Glycopeptide from Galeorhinus galeus,

The protocol developed for fractionating Squalus proteolytic digests was simplified in the case of the Tope. The freeze-dried dialysate from the digestion was followed by gel filtration to remove the contaminating protein, and trials proved Sephadex G150 the most effective column for this. The next stage of ion exchange chromatography was omitted after small scale trials, as the material was found to be uncharged. Subsequent analytical work was performed on this Sephadex G150 purified material. Purification on the Sephadex G150 columns is illustrated in Fig.34.

Columns, 30 x 2.5cm, were used, and were eluted with 1% NaCl. Fractions (5 ml) were collected and analysed for protein and carbohydrate.

## Analytical Methods

### UV absorption

A 1% solution of the dialysed freeze-dried jelly was scanned in an SP 500 spectrophotometer between 240 nm and 310 nm. in 1 cm silica cells

### Preliminary qualitative analysis of the material.

A Molisch test was carried out to check for the presence of neutral carbohydrate material. To a small volume (0·1 ml) of a 1% solution of the jelly in a 5 mm diameter tube, a few drops of 1% α-naphthol in ethanol were added. Concentrated $H_2SO_4$ was carefully poured in to form a layer on the bottom. The presence of a strong purple ring at the junction, developing after a few minutes, was taken to indicate a furfural-producing residue.

### Metachromasia.

The crude jelly from the wide range of species examined as part of the comparative study herein, and the chromatographic fractions from the two species studied in more depth were examined as to their ability to affect the well-known bathochromatic shift in the absorption maxima of the dyes of the thionin group. Azur A was used, and for routine examination, a 1% solution was made up in $^{M}/_{100}$ HCl. White ceramic spotting plates were used, and the change in colour from blue to purple observed by mixing equal volumes ( 0·05 ml) of dye solution and effluent. In some cases, the volumes were increased to a total of three ml, so that the resulting colour could be analysed spectrophotometrically. One gram of the crude jelly was dissolved to make 10ml in water, and 1.5 ml mixed with an equal volume of the acid dye solution.

Acid Hydrolysis of the Glycopeptide

For investigation of the component sugars known to be present following a positive result in the Molisch test, the polysaccharide components were investigated both quantitatively and qualitatively following acid hydrolysis. For qualitative investigation, solutions were made 1 M with respect to sulphuric acid and heated in a sealed tube for five hours at 105°C. The tubes were cooled, opened and the contents neutralised by adding barium carbonate. The suspension was filtered and the precipitate washed with hot water and the combined washings and filtrate concentrated for analysis on a rotating film evaporator.

For quantitative analysis of hexosamine components, the polysaccharide was hydrolysed under test conditions in two different concentrations of hydrochloric acid, 2 M and 4 M. The solutions were sealed vacuo and hydrolysed at 100°C for varying periods of time prior to assay for hexosamine, using the method of Gatt and Berman (1966) ( Fig. 2 ).Total hexosamine was also estimated by the technique of Boas (1953). (For the methodology of these assays, see "Quantitative Methods").

## Paper Chromatography

In order to simplify interpretation, a preliminary separation of neutral and amino sugars was performed. The neutralised hydrolysate from 10mg of glycopeptide was applied to a Zeokarb 225 ion exchange column (200-400 mesh, 4% divinyl benzene, 20 x 2 cm). The neutral sugars were eluted with water, and were located in the eluate by analysing 0.1 ml aliquots of the fractions for hexose by the method of Trevelyan and Harrison (1952). Amino sugars were then eluted with 0.1M $H_2SO_4$, and this fraction then neutralised with barium carbonate, and then concentrated.

Neutral sugars were chromatographically separated using ethyl acetate : propan-1-ol : water (7:1:2 by volume) as the mobile phase on Whatman No. 1 paper. Standard sugars were used as markers, and glucose, galactose, mannose, glucuronic acid, fucose, xylose, arabinose and lyxose were employed for this purpose. The amino sugars were identified by the method of Stoffyn and Jeanloz (1954) which involves reacting the amino group with ninhydrin. This gives an aldehyde which is the pentose corresponding to the original hexosamine. Chromatography in butanol : pyridine : water (5:3:2) formed the final step in the identification.

To check for any losses of sugars during these procedures, chromatography was carried out on the original neutralised preparation without any ion exchange column treatment, using ethyl acetate : propanol : water, as above.

The chromatograms were dried in a warm air oven at 45°C and the sugars detected by treating with the following reagents :-

(a) Alkaline silver nitrate - used according to Trevelyan et al (1950).

The chromatograms after development were fixed by dipping in 10% sodium thiosulphate solution, washing with a small amount of water, and drying

(b) Aniline hydrogen phthalate - according to Partridge, (1949).

Electrophoresis on paper

This was carried out on the glycopeptide material after digestion and purification as described, and 2.5 cm. wide strips of Whatman No.3 paper in 0·1M Veronal buffer pH 8·6 were used as support. Electrophoresis was carried out at 15 volt/cm for 90 minutes. The strips were dried in an oven at 100° and stained in 1% Azur A in 0·01 M.HCl. Excess dye was washed off with dilute acetic acid. Ox nasal septum chondroitin sulphate (a mixture of the isomers prepared by papain digestion) was used as a marker substance on a parallel strip and also mixed with the samples of Lorenzan glycopeptide.

## Preparation of Derivatives of the Neutral Sugar Reacting Components

To confirm chromatographic findings, attempts were madeto form derivatives from the neutral monosaccharide material. The 1 M. $H_2SO_4$ hydrolysate was freed from amino sugars on a Zeokarb 225 ($H^+$ form) column and the neutral component eluted with water. The material was concentrated to near dryness in vacuo at 50°C and taken up in 1·0 ml water. An aliquot derived from 25 mg of purified glycopeptide was used for derivative formation, and 0·5 ml of solution containing this was mixed with 0·5 ml of ethanol, 0·5 ml of $\alpha$-methyl-phenylhydrazine and 0·05 ml of 50% acetic acid. The solution was kept for 6 hours at 37°C and then at 0°C overnight. The crystals formed were washed with absolute ethanol and ether and dried in air. Standard and mixed melting points were made on the preparation, and on an authentic galactose $\alpha$-methyl-phenylhydrazone prepared in the same way.

## Nature of the Enantiomeric Form of the Galactose Component Found.

About 5 mg glycopeptide material from the main purified fractions of both Squalus acanthias and Galeorhinus galeus was hydrolysed. 2 N $H_2SO_4$ at 100°C for six hours was used, followed by neutralisation with barium carbonate and separation from the basic sugars as before. The material was concentrated, made up to 5 ml and 0·5 ml aliquots taken for the galactose oxidase assay of Avigad et al. (1961). An equal volume of reagent was added, and the system incubated at 30°C for three hours. Total galactose was assayed using the cysteine-sulphuric acid assay, (see Section on Quantitative analysis) with D-galactose as a standard solution and the results on these aminosugar-free solutions compared with those found in the galactose oxidase assay.

Infra Red Studies on the Glycopeptides.

About 2 mg of purified glycopeptide samples were dried in a dessicator over phosphorus pentoxide and sodium hydroxide in vacuo. 200 mg dry Analar KBr was ground with this, and a pellet made under pressure with a vacuum pump connected to the system. Two different instruments were used on different occasions (A) a Perkin-Elmer 472 and (B) a Perkin-Elmer Model 257. The difference is mainly in the presentation of results - the one plotting % Transmittance against wavelength, the other Absorbance against wavenumber.

Quantitative Assay Methods

Protein Chromatographic Analytical Methods

1) For the initial glycopeptide purification, the simplest analysis was used, as the main protein matter being separated at this stage was the proteolytic enzyme added, together with the smaller peptide material generated in the digestion process. For this, on the G 75 and G 100 columns (Fig. 8) O.D. was measured at 280 nm in the ultra violet. Generally for this sort of work, no calibration was used.

2) Lowry et al (1951) Protein Estimation

A more sensitive assay for proteins in chromatographic scans was used when the above method was inadequate, and a standard series of solutions 5-50 ug of bovine serum albumin used as a rough index of the level of protein present in the fractions. This method is a modification of the earlier Folin & Ciocalteau (1927) method, and relies on the presence of residues such as tyrosine for chromophore

formation and is, at best, a fairly rough guide to the actual amount of protein, although its fairly high sensitivity means that it is useful in allowing fractionation decisions to be made.

Coomassie Brilliant Blue Protein Assay

Recently, Bradford (1976) published a most useful technique which has largely replaced the Lowry protein method, which has the advantages of being much simpler to perform, and not less sensitive generally than the earlier more complex method. The Bradford method has the additional advantage that it is little affected by the presence of cations such as $Na^+$ or $K^+$, and in particular with relevance to this subject, it is little affected by the presence of carbohydrate. The dye binds to the protein, and the binding involves a substantial shift in the absorption maximum of the dye, from 465 nm to 595nm. This method of dye binding would seem to involve something akin to metachromatic shift already mentioned in polyanion binding but in this case the binding involves a shift to the longer wavelengths.

Methodology. Coomassie Brilliant Blue G-250 (Sigma).

For chromatographic analysis 0·1 ml of the protein solution was pipetted, and 1·0 ml of the protein reagent added. The mixed solutions were measured after two minutes and within one hour. For large numbers of fractions, analyses were performed in batches which could be read within the hour, and a few samples were measured twice, i.e. with each batch. The absorbance was measured at 595 nm against a reagent blank using 0·1 ml of the appropriate buffer + 1·0 ml of the reagent. The reagent performance was monitored by calibrating it with bovine serum albumin.

Nitrogen Determination

Nitrogen determination was performed on the wet jelly material by digesting measured aliquots of solutions in a Kjeldahl tube to which was added a small amount of dilute sulphuric acid. When the volume was reduced to 1·0ml., 1ml of concentrated sulphuric acid was added with selenium catalyst. The heating was continued gently until all water was driven off, and the tubes were then heated strongly for four hours. The distillation stage was performed in a Markham-type still. A more convenient and sensitive method for measuring nitrogen on limited amounts of material in resolved fractions was used more recently when a suitable electric heating block became available. The method is that of Jaenicke (1974) and involves using perchloric oxidation of evaporated aqueous samples in pyrex test tubes. The block temperature was maintained at 215°C and all the holes in the block were filled with test-tubes, irrespective of the number of samples, as this proved to be the only way in which reliable temperature control could be achieved. Aliquots of samples in solution (excepting those in tris-buffer) were pipetted to give 10 ug protein, and 25 ul perchloric acid was added. After heating to remove water, the tube was covered by a glass marble and heated 20 minutes. On cooling, 500 ul of water was added. The chromogenic reaction is as follows :-
To the above digest was added 0·5 ml of phenol reagent (2% phenol + 0·01% sodium nitroprusside in water) and 0·2 ml alkaline hypochlorite (0·02 M NaOCl in 2·5 M NaCl). The solutions were mixed on a whirl-mixer and kept 20 minutes at room temperature before reading at 578 nm. Standard graph prepared with Analar ammonium sulphate ( Fig. 4 ).

Neutral Carbohydrate Analysis

Carbohydrate was determined according to the anthrone method of Trevelyan & Harrison (1952) as modified by Dische (1955). Aliquots (0.5 ml) followed by 1.0 ml $H_2O$ were layered carefully on pre-cooled test tubes containing 2.5 ml anthrone reagent ( 0.16% freshly dissolved in conc. $H_2SO_4$).

The solutions were left at room temperature for 15 minutes and the absorbance at 640 nm read. Standard galactose solutions were used to calibrate. (Fig 3).

Neutral Sugars - Phenol Sulphuric Acid

A slightly more convenient method for neutral sugars was found to be that according to Dubois et al (1956), and this had the merit of giving more reproducible calibration curves, especially in salt solutions. Aliquots (0.5 ml) of sample were again used, followed by 0.5 ml of a 5% phenol solution, then 2.5 ml conc $H_2SO_4$. The mixture was shaken in a whirl-mix and left at 25°C in a water bath for 20 minutes before reading at 490 nm. Galactose standards were used, (Fig 5).

Fucose Determination

Gibbons (1955) published a method in which the chromophore formed was more stable than that reported for the cysteine hydrochloride method, and which had also more sensitivity. Solutions (1 ml) containing methylpentose (10 - 40 ug) were pipetted into a test tube, and 4.5 ml (6:1; v/v $H_2SO_4$:$H_2O$) added slowly with cooling in ice. The tubes were allowed to warm to room temperature and transferred to a boiling water-bath for 10 minutes. To these solutions after cooling was added 0.1 ml of 3.3% thioglycollic acid solution with shaking.

Absorbance was read at 415nm. (Fig 7). The difficulty with this method for the purposes of this study, is the interference by hexoses at concentrations twice that of fucose. Similar strictures apply to the cysteine/$H_2SO_4$ method.

Uronic Acid Assay

Uronic acid was determined in solutions of the purified material by the method of Bitter & Muir (1962). Standard graphs (Fig.6) were prepared using D-glucurone as a reference standard. The behaviour of D-galactose was also examined in this assay, because of possible interference from this source, and the spectra of the chromophores derived from galactose and from glucurone at 10% of the galactose concentration are shown (Fig. 27)

Procedure: Sodium tetraborate, ( 5.0 ml of 25mM ) in conc. $H_2SO_4$ was cooled in stoppered test tubes to -20°C. 1.0 ml of sample or standard was layered on top. The tubes were shaken gently in an ice bath during the process. The tubes were then heated at 100°C. for 10minutes, and cooled to room temperature. 0.2 ml of 0.125% w/v carbazole in ethanol was added, and the tubes were heated for a further 15 minutes at 100°C. After cooling, the absorbance was measured at 530nm.

Estimation of Total Hexosamine

The method used for analysing all the wet jellies was that of Boas (1953). Hydrolysis conditions for solutions of the jelly were 2N HCl at 100°C for 15 hours in sealed tubes, final volume, 2.0 ml. After this, the solutions were filtered into 10 ml. flasks and made up to the mark. Ion exchange columns of Dowex 50 (200- 400 mesh, 4.5% D.V. benzene) were prepared by pipetting a

1 : 1 ($^{w}/_{v}$) slurry into 20 x 1 cm tubes. Four ml of the diluted hydrolysate was pipetted twice on to duplicate columns for both samples and standards. (Glucosamine - HCl was used as the standard). The column was then washed with 10 ml water, and the effluent discarded. The hexosamines were then eluted with enough 2 M HCl to enable 8·0 ml (not 5·0 ml as in the paper) to be collected in a 10 ml measuring cylinder. One ml aliquots were transferred to 10 ml calibrated flasks for colorimety. A reagent blank was used in which the same protocol was followed subsequent to hydrolysis. The contents of the flasks were neutralised with 4 N NaOH to phenolphthalein, and the red colour was discharged with the minimum of 0·5 N. HCl. As the standards and blank were treated in the same way, there was no need to add the 4 M NaCl mentioned in the Boas account to equalise the salt concentrations.

Exactly 1 ml acetylacetone (4% $^{v}/_{v}$ in 0·75 M $Na_2CO_3$) was added and the flasks heated at 90°C for 30 minutes. The flasks were cooled for 5 minutes in a water-bath at room temperature when 2·5 ml ethanol were added with mixing, followed by 1·0 ml Ehrlich's reagent (2·67% $^{w}/_{v}$ p-dimethylamino benzaldehyde in 1 : 1 ethanol : HCl. The flasks were made up to the 10 ml mark with ethanol and the absorbance measured at 530 nm after 25 minutes.

Acetyl acetone solutions were made up freshly for each analysis series, the acetyl acetone itself being redistilled every few weeks.

The p-dimethylamino benzaldehyde was purified by dissolving BDH reagent in 2 M HCl, adding an equal volume of water, and 4 M NaOH added slowly with stirring; 150g batches were so treated and the

first 20 - 30 g to be precipitated were coloured At this stage the precipitate was filtered off and discarded. The remaining precipitate was almost white and was collected. The collection was stopped before neutralisation was complete. Yield from 150 g, 100 g

Gatt and Berman Method (1966)

This method was used exclusively after trials although much of the earlier results were derived from the above method adapted from Boas. The Gatt and Berman method was more sensitive and more reproducible, certainly when applied to the Lorenzan glycopeptides. No significant modifications from the published method were used, and the finding of a slightly different calibration curve for glucosamine compared with galactosamine was confirmed; despite this, the method was used for total amino sugar analysis (Fig. 2 ).

Analysis of Neutral Sugar Components by Gas Liquid Chromatography.

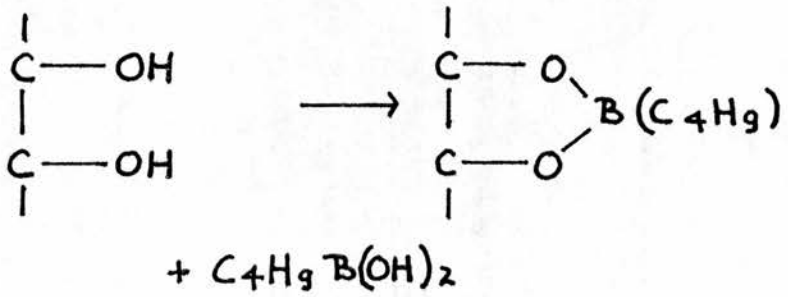
Both qualitative and quantitative analysis of neutral sugars amino sugars and uronic acids can be performed using G.L.C. However, it is possible only in theory to perform amino sugar analysis simultaneously with neutral sugar analysis, as the hydrolysis conditions for the two types are different. For neutral sugars, the alditol acetate method as described by Crowell & Burnett (1967) was tried and found to be useful. While methods have been described (Griggs et al 1971) for quantitating amino sugars by alditol acetates, in this study the method was found to be unsatisfactory. Apart from column bleed at the temperature required to elute the hexosamine derivative,

varying responses were found between runs, probably due to oxidation of these derivatives at this temperature on the column. This cause was suspected when the carrier gas used, B.O.C. oxygen-free nitrogen, was found to have variable amounts of oxygen present as determined by an oxygen electrode. The authors of the paper used helium, a gas which is too expensive for routine use.

In the method of Crowell & Burnett, the columns were 6', packed with 3% ECNSS-M on Gas Chrom Q (Phase Separations Ltd.), and run isothermally at 180°C. At these temperatures, the neutral sugar derivatives behaved consistently. Flow rates of 45 ml/min were found suitable for the nitrogen carrier gas used. A PYE 104 gas chromatograph was used. The internal standard used was mannitol. Hydrolysis conditions for the Lorenzan glycopeptide were M HCl 6 hours, 105°C in sealed tubes in vacuo. The HCl was removed over NaOH and phosphorus pentoxide in a dessicator at room temperature. The reduction was with sodium borohydride (2 mg in 1·0 ml., final volume) at room temperature for two hours. Excess $NaBH_4$ was destroyed by adding drops of glacial acetic acid until gas evolution stopped. The solution was then freeze-dried, and the borate formed removed prior to acetylation by three methanol extractions to remove the volatile methyl borate. Acetylation was with 0·2 ml each of dry pyridine and acetic anhydride in a screw capped glass bottle with a Teflon liner in the screw cap, at 105°C for four hours. The pyridine and acetic anhydride were removed on a rotating film evaporator at 40°C, and as internal standards were present, no special care was needed to transfer quantitatively. For chromatography, samples were then dissolved in dichloroethane, and 2 - 10 ul samples injected into a heated (210°C) port. (Fig. 8a)

GLC of Butyl Boronate Derivatives

Eisenberg (1972) published a method of GLC fractionation based upon the formation of diol alkaneboronates:-




which works well for compounds with an even number of -OH functions such as pentoses (4 OH) but not for hexoses (5 OH), but does well with hexitols (6 OH). Since hexosamines can be reduced by borohydride to hexosaminitols, the method can be made to work for these difficult sugars.

The graph ( Fig 1) shows the reults of a typical neutral sugar separation performed in this way. The derivatives were formed by reducing 1mg of each sugar dissolved in 1.0ml water for one hour with 10mg sodium borohydride. The excess borohydride was destroyed by adding a drop of acetic acid. The solution was dried by lyophisation, and then 2ml portions of methanol added and evaporated off four times by a water pump to remove the borate formed as methyl borate. The boronates were then formed from the alditols thus created by adding 5mg of butyl boronate in 1 ml pyridine. Gentle warming for five minutes dissolved the material, and the preparation was ready for injection.

The instrument used was a Pye 104 Gas chromatograph and the analysis was performed using nitrogen, flow rate 35 ml/min, as the carrier gas. The temperature was 200°C, and the column was OV 17 on Gas chrom Q. For neutral sugars the column was two metres long. The most important use of GLC, however, was in the analysis of the individual amino sugars. As their derivatives are less volatile, the column length was increased to six metres by joining two 3 metre columns. The temperature in this case was increased to 240°C.

Generally, about 2 - 5ug of total sugars in 5 - 10ul was used per injection. Reproducibility was found to be well within 5% on measuring the area of the curves by multiplying the height by the width at half height.

Separation and Analysis of the Amino Sugars by the method of Gardell.

Gardell (1953) published the first adequate method for routine analysis of the individual hexosamines, based on their separation on a Dowex 50 ion exchange resin column, their elution with 0.3 M HCl and subsequent assay of the fractions using an Elson-Morgan colorimetric reaction. Gardell's method is more variable than could be desired, requires a great deal of attention to detail in the colorimetry to produce acceptable levels of reproducibility, and is a time-consuming method

of analysis. The method of sealing the tubes during the colorimetric reactions was found to affect the colour yield (Fig. 9 ) and separate calibration graphs were required for the two hexosamines.* The method of sealing of the tubes finally used was with parafilm which was kept from melting by the heat from the water-bath by a flow of air from a fan directed on top. The concentration of sodium carbonate was increased from 1·25M to 1·5M which resulted in increased sensitivity without any obvious loss of reproducibility.

In most of the earlier analysis of the Lorenzini jellies, the comparative studies were performed by this method which took about 24 hours per analysis, as was the analysis of the cartilage material. The later results, (the analysis of the purified fractions of the Lorenzini glycopeptides) were performed using the more convenient and less time-consuming gas-liquid chromatographic method.

Analysis of Acetyl Groups.

The method of Ludowieg & Dorfman (1960) was used. This depends upon deacetylation of N-acetyl compounds with 2 M. HCl-methanol. The acetyl groups are converted into methyl acetate which is determined colorimetrically as an hydroxamic-ferric complex. As the range is over 1-10 u moles, the sensitivity of the method is suitable for small amounts of sample. The HCl gas for the deacetylation was commercial gas from a cylinder (B.O.C.) which was further dried via a concentrated sulphuric acid wash. The methanol was distilled from molecular sieve drying agent and stored over the same reagent. For standards, 5 mM ethyl acetate in methanol-$H_2O$ (50:50 v/v) was used, and all measurements were performed against an HCl-methanol blank. The method

* For routine purposes, a correction factor was used for galactosamine colour yield, and glucosamine only was used.

was checked against synthetic crystalline N-acetyl glucosamine (Fig. 10 ).

Sulphate Analysis

The method finally adopted was that of Jones & Letham (1956). Most sulphate analytical methods are laborious and of low sensitivity. A simple and fairly sensitive method according to Inoue (1965) was tried. This method uses trypaflavin (2,8 - diaminoacridine hydrochloride), but the method was found to lack reproducibility. The method of Dodgson (1961) was adequate in this respect, but suffered from a lack of sensitivity for the purposes required. Several methods have been described based on precipitation of the sulphate as an insoluble salt after hydrolysis, and redissolving a chemically modified salt. Kent & Whitehouse (1955), Antonopoulos (1962), used methods based on this principle, and that used here by Jones & Letham is similar. All such methods suffer from the consequences of the slight solubility of the precipitate which results in error at the low end of the assay.

The solubility of the 4-amino-4$^{1}$ chlorobiphenyl sulphate was allowed for in the calibration of standard sulphate curves. An inverse calibration curve was obtained, maximal absorbance being at lowest sulphate concentrations, or a positive slope can be obtained by subtracting all readings from the high absorbancies corresponding to zero sulphate. The hydrolytic conditions used were 4 hours in 4N HCl at 105$^{o}$C.

Enzyme Treatment of the Lorenzini glycopeptide from Squalus acanthias (digest purified on G 100)

The purified polysaccharide was incubated at 37°C for 5 days, 15mg in 1 ml using toluene as a preservative with 2·5 mg. testicular hyaluronidase (B.D.H.). The sample was dissolved in 0·1 M acetate buffer (pH 6·5), 0·15 M with respect to sodium chloride. The experiment was performed twice, at different times, and with different glycopeptide preparations. A control was performed in which the incubation was performed with hyaluronidase inactivated by heating in a boiling water-bath for 30 minutes. The activity of the enzyme was checked against a preparation of chondroitin sulphate (B.D.H.). The digested material in all cases was fractionated on a column of Sephadex G-75 (22 x 1·7 cm). The entire sample was applied and the column eluted with water. 1·0 ml fractions were collected, and the eluate examined by the Molisch test, and by the metachromatic activity towards Azur-A at pH 2·0. In the digested (and control) samples of Lorenzini, the metachromatic fractions were pooled into two lots arbitrarily divided for analysis. Fractions 6-16, and fractions 17-28 were taken for quantitative amino sugar analysis by Gardell's (1953) method. The small molecular material, (judged by chloride tests) started at fraction 30.

Amino acid analyses of the Lorenzini Jelly and the derived glycopeptides.

Amino acid analysis was performed on the original jelly material using a commercial automatic amino acid analyser (Locarte Model 1V) Standard amino acid mixtures (25 n moles each acid) were used as controls. The system was automatic, based on the ion-exchange method of Moore & Stein (1951, 1954).

A sample (2 mg) of the original jelly was taken for hydrolysis, and a 10% aliquot of this applied to the column. Hydrolysis conditions were 6 N HCl containing 0·01% phenol in vacuo 24 hours 110°C. The samples applied were in solution, an equal volume of conc. HCl added, and the neck of the test tube constricted by heating. The solutions were then frozen in alcohol-solid $CO_2$ mixtures, and allowed to thaw. They were frozen once more and then pumped down on an oil pump. Whilst still evacuating, the tubes were sealed. After hydrolysis the tubes were spun at 3000g for 5 minutes, opened 15 mm from the bottom to give a small cup for ready evaporation of the HCl and pumped down in a dessicator containing solid NaOH and phosphorus pentoxide overnight. This dry residue was taken up in the 1·0 ml of first buffer of the analyser, and 0·1-0·4 ml. aliquots applied to the column for chromatography. Quantitation was performed on the results relative to the standards by measuring the peak areas in arbitrary units (half the height x width at that point). Standards of both glucosamine and galactosamine hydrochlorides were used together with the synthetic amino acid, $\alpha$-amino butyric acid. The amino sugars were again run, following treatment with the hydrolytic conditions used for the amino acids, in order to appreciate their position in the amino acid analyser effluent, and the consequences of their destruction.

Alkaline degradation of the purified glycopeptide of both Squalus acanthias and Galeorhinus galeus.

To study the linkage region, the effect of dilute alkali on Lj S.a. G 100 and on Tope G 150 was investigated.

Approximately 20 mg was weighed out and dissolved in 3.5 ml water. From these solutions, 3 x 1.0 ml aliquots were taken and treated as follows :-

A. The sample aliquot + 1.0 ml M NaOH, was flushed with nitrogen gas and kept 45 hours at 25°C. This was followed by neutralising with HCl and the solution was made up to 5.0ml.

B. The sample aliquot was treated with 1 ml 2 M $NaBH_4$ for 20 minutes at 25°C, and 2 ml M NaOH were then added and the tubes flushed with nitrogen prior to stoppering and incubating at 25°C for 45 hours. The tubes were opened and neutralised carefully as before. The material was then freeze-dried, and 5 x 3 ml aliquots of methanol added, with evaporation at each addition, at 45°C in a rotating film evaporator to remove borate as the volatile methyl borate. The material was re-dissolved in 5.0 ml $H_2O$.

C. The solution (1.0 ml) was treated with 1.0 ml NaOH. This solution was neutralised immediately and made up to 5.0 ml.

From (A), (B) and (C), aliquots were removed for analysis as follows :-

1) An aliquot (0.5 ml) for amino acid analysis

after hydrolysis in 6 N HCl for 24 hours, 110°C.

2) An aliquot (0.5 ml) was hydrolysed in 2 M HCl (an equal volume of 4 M HCl was added), in a sealed tube under nitrogen for 15 hours at 105°C. This fraction was then analysed for amino sugars by the butyl boronate method (Eisenberg, 1972). The above refers to samples(A) and(C) only.

3) An aliquot (0.5 ml) was tested for preformed Morgan-Elson chromogen by a modified Reissig assay (Reissig et al., 1955). The modification involves omitting the heating stage after addition of the potassium tetraborate. N-acetyl glucosamine was used as a standard in this assay. A calibration graph is shown using this material, (Fig 11).

4) Aliquots (1.0 ml) of(A) and(C) were fractionated on a Biogel P2 column. Fractions (1.0 ml) were collected and analysed by the phenol-$H_2SO_4$ neutral carbohydrate assay.

5) Aliquots were measured before and after alkaline treatment for absorption in the ultra-violet region at 241 nm.

Reissig Assay (1955) for N-Acetyl Amino Sugars.

Standard (50 ug/ml) solutions of N-acetyl glucosamine were used to assay for this. To the samples ( alkali-treated glycopeptides), blanks and standards (0.5 ml), was added 0.1 ml 0.8 M potassium tetraborate. The standards were heated in a boiling water-bath for exactly 3 minutes and then cooled. The p-dimethylaminobenzaldehyde reagent (3.0 ml) was added and the tubes incubated at 37°C for 20 minutes. The absorbancies were read at 585 nm. A calibration graph using standard solutions of N-acetyl glucosamine, and the spectrum of the chromophore are shown (Figs. 11 and 12).

Biogel P2 Chromatographic Studies on the Alkaline Degradation of Lorenzini Glycopeptides from Squalus and Galeorhinus.

Biogel P2 columns 70 x 2 cm were used to investigate chromatographically any changes in size distribution of the glycopeptides during alkaline degradation. Columns were eluted with 1% saline, and calibrated with dextran blue, sucrose and glucose (Fig 28a). The glycopeptide, LS/S.a. G100 (30 mg) was applied in 2.5 ml of 1% saline, and eluted with 1% saline (Fig 28b). Fractions (1.5 ml) were collected and analysed by the Phenol/$H_2SO_4$ reaction on 0.5 ml aliquots. The experiment was repeated after alkaline degradation performed as previously, and this time fractions were also analysed for the presence of Morgan-Elson chromogen qualitatively (Fig 28b). The same procedures were also applied to Tope glycopeptide material (Fig 28c).

## Cartilage in Elasmobranchs compared with mammalian cartilage with respect to ageing

### Experimental

Preparation of cartilage from Squalus acanthias

All fish were weighed and measured on a measuring board after trawling. The entire pectoral girdle was dissected out and used for this study. The pair of small morphologically distinct pieces of cartilage containing calcified rings about 3-5 mm diameter was removed. This structure occurs in all fish, irrespective of size. Perichondrial tissue was also removed. Entire girdles, or in the case of larger fish, symmetrical half girdles, were taken and cut by hand into thin slices and dehydrated for several days in acetone with frequent changes of solvent. This dried material was ground to a fine powder and dried to constant weight over phosphorus pentoxide.

### Chemical Analysis

Uronic acid was estimated on the finely ground material by the method used by Shetlar & Masters (1955).

Total hexosamine was estimated by Boas (1953) method after hydrolysis in 4 M HCl for 14 hours at 105°C. After weighing out aliquots for this assay, the samples were kept overnight in their hydrolysis tubes in 1.0 ml of water, and prior to hydrolysis, an equal volume of 8 M HCl was added, when the tubes were evacuated and sealed. The ratio of the two amino sugars was found by separation on ion-exchange columns according to Gardell's (1953) method. All measurements, including the Gardell analyses were performed in duplicate, and repeated when differences above 5% were found in the

duplicates.

Ash was determined by the weight differences after heating to 500°C in an electric furnace to constant weight. A few drops of M $H_2SO_4$ were added previous to strong heating to convert any carbonates to sulphates.

Digestion with Proteolytic Enzymes

Pieces of fresh cartilage from the older (larger) animals were homogenised in a Buhler high speed homogenisor at 50,000 RPM with cooling for intermittent periods in water. The homogenate was autoclaved at 10lbs to clear it, and filtered. One gram aliquots of wet tissue so prepared were treated with 10mg collagenase at 37°C 24 hours. After digestion, the solution was dialysed 24 hours against water. The digest was scanned from 240-300 nm, and the pH adjusted to 6·0 with HCl, the extract heated at 56°C for two hours, and the uv-scan repeated.

Sialic Acid Assay

Aliquots of the homogenate were made 0·05 M with sulphuric acid and heated at 80°C for one hour. Thereafter, sialic acids were assayed by the method of Svennerholm (1958).

Comparison with mammalian cartilage.

Using samples of horse nasal septum (material from Dr. J.A. Szirmai) fractionated into layers in the histological classification of Szirmai & Doyle (1959) based on dye-binding in young (two layers) and old (three layers) animals, sialic acid was determined on the homogenates of material prepared.

Samples were also treated with collagenase, analysed in the u.v. as for the elasmobranch material, followed by heating at pH 6.0 for 2 hours at 56°C, and repeating the analysis.

Digests of this material were also treated with pepsin-trypsin at pH 2.0 for 24 hours and pH 7.0 for 24 hours followed by dialysis against running water. Analysis in the u.v. of this digested material was also performed. ( see 'Results' Section).

# CHAPTER THREE

## EXPERIMENTAL RESULTS

### Preliminary Examination

### u.v. absorption

The jelly from Squalus acanthias was soluble in water with shaking, and in the u.v. showed a complex peak containing absorption in the region of 250-275 nm (Fig. 13 ) with no definite 280 nm maximum characteristic of protein, and in Squalus material, a maximum at 266 nm exists. Fig.14 shows the spectrum of Galeus material.

### Metachromasia.

All the jellies studied showed the characteristic bathochromatic shift, excepting for that of Galeorhinus galeus. Fig. 15 shows the results obtained for Squalus acanthias and Galeorhinus galeus with Azur A. The results for Galeorhinus galeus are indistinguishable from those with the dye alone (Fig. 16 ) under these conditions, i.e., a single maximum around 630 nm, whilst Squalus acanthias material shows, in addition, a maximum around 540 nm accounting for the purple colouration.

### Molisch Test.

This was strongly positive in solutions from all sixteen species examined here, including the non-metachromatic one.

### Chemical Analysis of the Crude Jelly Solutions.

Table 3 summarises the information for nitrogen (Kjeldahl), total hexosamine (Boas, 1953), the amino sugar ratios (Gardell, 1953), uronic acids (Bitter and Muir, 1962) and hexose (Trevelyan etal 1952). The animal groupings to which the species

Table 3 Chemical Analysis of the Dissolved Jellies from varying species; metachromatic behaviour of the material towards Azur - A at pH 2.0.

| Species | Nitrogen µg/mg | Total Hexosamine µg/mg | % Total | | Uronic Acid µg/mg | Hexose µg/mg | Metachromasia at pH 2.0 | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Glucosamine | Galactosamine | | | | | Type |
| Carcharinus glaucus | 6.38 | 9.6 | 60.0 | 40.0 | 0.55 | 9.82 | + | large shark | shark-like |
| Cetorhinus maximus | 13.8 | 13.8 | 57.1 | 42.9 | 0.58 | 31.5 | + | large shark | shark-like |
| Galeorhinus galeus | 6.2 | 10.9 | 46.5 | 53.5 | 0.43 | 12.2 | - | large shark | shark-like |
| Galeus melanostomus | 3.48 | 6.17 | 84.5 | 15.5 | 0.36 | 7.24 | + | | shark-like |
| Scylliorhinus canicula | 1.81 | 7.8 | 71.0 | 29.0 | 0.42 | 10.7 | + | | shark-like |
| Scylliorhinus stellaris | 4.82 | 15.12 | 65.6 | 34.4 | 0.87 | 17.9 | + | | shark-like |
| Squalus acanthias | 7.58 | 10.5 | 80.0 | 20.0 | 0.58 | 11.6 | + | | shark-like |
| Raja batis | 6.0 | 3.7 | 79.0 | 21.0 | 0.93 | 4.75 | + | large ray | Ray-like |
| Raja brachyurus | 2.75 | 4.63 | 87.0 | 13.0 | 0.52 | 6.3 | + | | Ray-like |
| Raja clavata | 3.03 | 5.93 | 80.6 | 19.4 | 0.50 | 5.4 | + | | Ray-like |
| Raja microcellata | 3.65 | 4.4 | 76.1 | 23.9 | 0.56 | 4.92 | + | | Ray-like |
| Raja montagui | 2.80 | 6.2 | 83.3 | 16.7 | 0.76 | 8.63 | + | | Ray-like |
| Raja naevus | 2.45 | 5.7 | 87.6 | 12.4 | 0.40 | 5.92 | + | | Ray-like |
| Torpedo nobiliana | 6.07 | 10.3 | 58.7 | 41.3 | 0.45 | 10.0 | + | | Ray-like |
| Hydrolagus affinis | 2.78 | 2.44 | 11.19 | 88.81 | 0.15 | 2.29 | + | | Holocephalic |
| Chimera monstrosa | 6.81 | 3.2 | 20.5 | 79.5 | 0.16 | 1.50 | + | | Holocephalic |

belong is shown in the same table.

Paper Chromatographic Results.

In all sixteen species, the main, if not the only, neutral sugar detected by paper chromatographic methods in the solvent systems used, was galactose. In the ethyl acetate system, R glucose values of 0·87 were found, and 0·89 in the butanol-pyridine -water system.

Table 4 Solvent:- Propanol-Ethyl Acetate - $H_2O$

| Standards. | R - glucose |
|---|---|
| Glucose | 1·0 |
| Galactose | 0·87 |
| Fucose | 1·31 |
| Mannose | 1·11 |
| Glucuronic Acid | 0·27 |
| Ribose | 1·63 |
| Glucosamine | 0·68 |
| Galactosamine | 0·63 |

Table 5 Solvent:- Butanol - Pyridine - $H_2O$

| | R - glucose |
|---|---|
| Glucose | 1·0 |
| Lyxose | 1·75 |
| Arabinose | 1·31 |
| Galactose | 0·87 |

In some species, notably Cetorhinus maximus, traces of material with R-glucose values corresponding fairly closely to fucose were sometimes found but the amounts were small and the observations not always repeatable, making it difficult to be certain of its real existence.

In Squalus acanthias, some slow moving material corresponding most closely to, but never exactly with, the D-glucuronic acid standards, was found, but again, always in trace amounts relative to all other sugars found in the hydrolysates. Doubt about the existence of this was never entirely resolved on the basis of this type of evidence.

Amino Sugars.

Both lyxose,(corresponding to galactosamine)and arabinose (corresponding to glucosamine)were found in the hydrolysates when the material was reacted with ninhydrin. In the chromatograms developed using the propanol solvent system, both amino sugars were found in hydrolysates from all species when the pretreatment with the sulphuric acid resin was omitted.

α - Methylphenylhydrazone Derivative.

The findings from the paper chromatographic investigations involving galactose as the main, or possibly the only, neutral sugar were confirmed by forming this type of derivative in hydrolysates from which the amino sugars, now known to be present, were removed. A melting point of 179° - 180° (uncorrected) was found for the crystals isolated, and these gave a mixed melting point of 179° - 181° C with crystals prepared from chromatographically pure D-galactose.

Enantiomeric Nature of the Galactose.

This was studied by enzymatic attack using D-galactose oxidase, which catalyses the oxidation of the primary hydroxyl group of D-galactose (Avigad et al, 1961), liberating an equimolar quantity of hydrogen peroxide which is estimated using peroxidase and the leuco (reduced) form of a dye (Huggett & Nixon, 1957). As any sugar with the correct D-galactopyranosyl configuration can act as a substrate

for this enzyme, including D-galactosamine, the amino sugars were first removed on small Dowex-50 columns.

The results compared with the hexose values found in terms of D-galactose by the method of Trevelyan & Harrison (1952) are shown in Table 6 :-

Table 6

| | Squalus acanthias | Galeorhinus galeus |
|---|---|---|
| | ug/mg | ug/mg |
| Hexose (chemical) | 11.6 | 12.2 |
| D-galactose (Enzymatic) | 10.2 | 10.4 |

Amino Sugar Analysis (Gardell) of the Crude Jelly Material

A calibration curve for this analytical method, using D-glucosamine - HCl standards is shown (Fig. 17 ). The typical separation of the two amino sugars is shown in (Fig.18 ), glucosamine being eluted with a peak maximum at around 58.0 ml, and galactosamine with a peak maximum at around 66.0 ml. Table 3 lists the findings for the entire sixteen species, showing variation between the species for glucosamine to galactosamine molar ratios of 7.06 : 1 (Raja naevus) to 0.126 : 1 (Hydrolagus affinis). In the two species studied here in most detail Squalus acanthias and Galeorhinus galeus, the ratios for GlcN : GalN were 4 : 1 and 0.87 : 1 respectively. Thus both 2-amino 2 deoxy D-hexoses are found in all specimens examined.

Preliminary Purification of Glycopeptide Material from the Lorenzini Jelly.

After digestion with papain, 5 ml aliquots of the concentrated digest containing the equivalent of 5g of the original jelly, purified on the G50 columns as described, showed one large non-symmetrical peak of metachromatic anthrone-positive material which came out with some protein, followed by a smaller molecular weight protein-peak (Fig. 19 ). The fraction volumes were 4·0 ml, and fractions 10-30 inclusive were pooled. The pooled material was concentrated to 10ml and precipitation of this with ethanol gave a fluffy whitish precipitate (150mg). Analysis of this material showed the following:-

Table 7 Chemical Analysis of purified Lorenzini-jelly polysaccharide preparation from Squalus acanthias (Figures for sodium salt)

| | % | Mole Ratio |
|---|---|---|
| Galactose | 30·0 | 1·0 |
| Uronic Acid | 2·85 | 0·08 |
| Total Hexosamine | 28·6 | 0·96 |
| Acetyl | 7·4 | 1·03 |
| Sulphate | 14·5 | 0·91 |
| Nitrogen | 3·8 | --- |

The position of the acetyl group was investigated on this material. Treatment of this with 0·05 M sulphuric acid at 100°C followed by neutralisation and buffering at pH5·0 with 0·2M acetate buffer and finally boiling with ninhydrin reagent for 10 minutes gave a marked blue colour with the acid hydrolysed material, and

none with a control which used sulphuric acid neutralised before heating. Gardell amino sugar analysis of this material showed a ratio of glucosamine : galactosamine close to 4 : 1.

Electrophoretic Behaviour of the Glycopeptide.

Paper electrophoresis of the digested material in veronal buffer at pH 8·6 showed a narrow metachromatically-staining band migrating at approximately half the rate of chondroitin sulphuric acid (deproteinised). Mixtures of chondroitin sulphate and the Lorenzini glycopeptide separated readily giving bands at positions corresponding to those of the unmixed substances. Lorenzini jelly material which had been dialysed against the veronal buffer did not migrate from the origin under these conditions. (Fig. 20 ). The experiment was repeated, four strips at a time on six occasions, and the hexosamine ratio on eluted bands (using thin strips from either end for staining to control the position) was measured by Gardell's (1953) method following dialysis against water and hydrolysis. The results are shown in Table 8 .

Table 8 Hexosamine analysis on Electrophoretically Purified Glycopeptide Material from Squalus acanthias

| | Glucosamine | Galactosamine |
|---|---|---|
| (1) | 78·8 | 21·2 |
| (2) | 79·7 | 20·3 |

The above results are expressed as a percentage of the total hexosamine.

## Effect of Hyaluronidase on the Purified Glycopeptide

The material after digestion was examined for evidence of degradation in several ways. The material applied to Sephadex G75 columns, similar to the G-50 columns used before with similar fractionation schedules, gave a similar single large non-symmetrical peak, and this material proved to be metachromatic at pH2·0, and also contained all the anthrone + positive material. The fraction volumes were again 4·0 ml, but in the G75 column, all the material which reacted for neutral hexose was eluted by fraction 28. The eluted material was pooled into two (arbitrarily) divided fractions, Fractions 6-16, and Fractions 17-28. Results of hexosamine analyses are shown (Table 9 ) :-

**Amino Sugar Analysis of Hyaluronidase Treated Glycopeptide.**

**Results expressed as percentage of total hexosamine.**

| | | Glucosamine | Galactosamine |
|---|---|---|---|
| Expt 1 :- | | | |
| Fractions | 6 - 16 | 74·4 | 25·6 |
| " | 17 - 28 | 82·5 | 17·5 |
| Expt 2 :- | | | |
| Fractions | 6 - 16 | 74·6 | 25·4 |
| " | 17 - 28 | 83·3 | 16·7 |
| Control:- Fractions | 6 - 16 | 77·1 | 22·9 |
| " | 17 - 28 | 81·2 | 18·8 |

The control represents the results obtained when the enzyme was boiled before the incubation.

90% of the material incubated with hyaluronidase was recovered in the macromolecular fraction. Analysis by infra-red of the two arbitrarily pooled fractions did not show any significant differences between the fractions, nor from the parent material used in the incubation.

## Infra - Red Analysis of the Glycopeptides

Orr et al (1952), and Orr (1954) published the first results of infra-red studies of mucopolysaccharides and Lloyd et al (1961) studied the infra-red spectra of polysaccharide esters and the effects of chemical desulphation on the absorption bands.

For purposes of comparison, a table has been drawn up (Table 10 ) of the main absorption bands of the common mammalian mucopolysaccharides (glycosaminoglycans) and of the main two glycopeptide preparations reported here, viz., those of Squalus acanthias and Galeorhinus galeus.

The spectra of these two preparations is shown in Fig. 23. Material from Squalus acanthias shows a strong absorption band at 1240 $cm^{-1}$. ( S = O stretching), and absorptions in the 800-950 $cm^{-1}$ region, characteristic of ester sulphate. These absorptions appear largely or entirely absent in the spectrum of purified material from Galeorhinus galeus.

In many other respects, the two spectra show similar features. They exhibit strong absorptions in the 1550 and 1640 $cm^{-1}$ region, attributable to N-acetyl residues. Ionised and non-ionised carboxylic acid functions do not appear significantly, judging by the weakness of absorptions in the regions 1736 and 1230 $cm^{-1}$ ($COOH$) and 1610 and 1410$cm^{-1}$ ($COO^-$).

Note:- Abbreviations used in table - 10

str. - strong
shd. - shoulder
vibn. - vibration
wk. - weak

Table 10 Glycosaminoglycans - I.R. Assignments

| Freq. $cm^{-1}$ | Squalus acanthias | Tope | Chon. 4-$SO_4$ | Keratan $SO_4$ | ASSIGNMENTS |
|---|---|---|---|---|---|
| | 680 | | | | |
| 700 | | | 715wk | | |
| | | 775wk | | | |
| 800 | | | | | |
| | 820wk | | | 825wk | |
| | | | 850wk | | |
| | 890wk | 875 | | | |
| 900 | | | | | |
| | | | 925 | | |
| | 940 | | | | |
| | | 975shd | | 950wk | |
| 1000 | 1000shd | | | | |
| | | 1035 | 1030 | | |
| | 1065str | broad | | 1065 | C – O str<br>C – C vibn<br>C – N str<br>common to all carbo-hydrates. |
| | | 1075 | | | |
| 1100 | | | | | |
| | | 1120wk | | | |
| | 1125shd | | 1125wk | 1125wk | |
| | 1150shd | | | | |
| | | 1160shd | | | |
| 1200 | | | | | |
| | | | | 1235 | 1230 C – O str in –COOH (wk) |
| | 1240str | 1240wk | | | 1240 S = O stretching in $-OSO_3$ |
| | | | 1250str | | |
| 1300 | | | | | |
| | 1315 | 1315 | | 1315wk | 1315 C – N str in $-NHCOCH_3$ |
| | | | | | 1375 $CH_3$ as in $-NHCOCH_3$ |
| | 1385str | 1380 | 1380 | 1385 | |
| 1400 | | | | | |
| | | | 1415 | | |
| | | | | 1420shd | 1410 C – O str in $COO^-$ |
| 1500 | | | | | |
| | 1550str | 1550str | | | |
| | | | 1560str | 1565str | 1560 N – H defn in $-NHCOCH_3$ |
| 1600 | | | | | |
| | | | | | 1610 C = O str in $COO^-$ |
| | | | 1625str | 1640str | |
| | 1650str | 1650str | | 1665shd | 1648 C = O str in $-NHCOCH_{33}$ |
| | | 1690shd | | | 1650 NH defn in $-NH_2$ |
| 1700 | | 1725shd | | | 1736 C = O in COOH |

775 – 1000: CS"C" equatorial $SO_4$

712, 725, 840, 852, 855, 928: sulphate in CSA and CSB

820, 928: C–O–S str.

The need for further purification of the glycopeptide.

During the experiments with hyaluronidase,(p50), amino sugar analysis gave the first indications of possible heterogeneity in the glycopeptide preparations. One check considered worth performing was amino sugar analysis (Gardell,1953) on the crude jelly from four individual animals. It seemed just possible that variations might occur between individual specimens. The results which are shown below( Table 11) do not lend support to this idea.

TABLE 11

Hexosamine analysis on native jelly from individual animals.

(Figures are expressed as a percentage of the total.)

| Animal | Glucosamine | Galactosamine |
|---|---|---|
| 1 | 80.5 | 19.5 |
| 2 | 78.8 | 21.2 |
| 3 | 79.0 | 21.0 |
| 4 | 79.7 | 20.3 |

Further purification of the glycopeptide

A further type of purification was attempted using a DEAE Sephadex A50 column, 40 X 2.5 cm equilibrated in 0.1 M Tris buffer, pH 8.0. Sephadex G50 purified material was applied(100mg) in 10 ml of buffer and eluted with 5 x 100 ml steps of increasing salt concentration from 0.25 M to 1.5M . Fractions of 10 ml were collected and analysed for carbohydrate. Two main peaks were observed, at 0.75 M and at 1.0 M NaCl (Fig 21) .

In the light of the heterogeneity shown in the DEAE Sephadex experiment( Fig. 21), experiments were designed to produce sufficient material for more detailed analytical study. The gel filtration experiments (p48) were repeated with some changes. Sephadex G100 columns (42 x 5 cm) were packed in and eluted by 1% NaCl solutions. Fractions of 10ml were collected and analysed for carbohydrate by the phenol/$H_2SO_4$ method (Fig 8) . One large peak of metachromatic material was obtained, and when the fractions containing this were pooled, dialysed and freeze-dried, 110 mg of the original 150 mg applied were obtained.

Analysis of the fractions for protein at 280 nm showed the presence of two further peaks, but neither was metachromatic and both were discarded. This experiment was repeated on twelve occasions in order to have sufficient stock of LS/G100 for further purification.

## Purification of LS/G100 on DEAE Sephadex

A column of Diethylaminoethyl Sephadex A50 was prepared, of dimensions 70cm x 6cm, packed in 0.1 M Tris/HCl buffer at pH 8.0. A sample of LS/G100 ( 850 mg) was applied and eluted with a total of 9 litres of buffer with salt steps varying from 0.4 M to 1.2 M. Fractions of 25 ml were collected and analysed at 490nm for neutral carbohydrate using the phenol/ $H_2SO_4$ method. Three carbohydrate reacting peaks were obtained (Fig. 22), and recoveries and designations are shown in Table 11a.

Table 11a Recovery of material from DEAE Sephadex Columns.

| | Salt Concentration | Recovery | Fraction |
|---|---|---|---|
| Peak 1 | 0.4 M | 280mg | LS/S.a/G100/DE-1 |
| Peak 2 | 0.6M | 470 mg | LS/S.a./G100/DE-2 |
| Peak 3 | 1.2M | 50mg | LS/S.a./G100/DE-3 |

Peak 1 did not show on analysis a symmetrical peak shape, but rather a diffuse peak indicating that the material was still to some extent polydisperse (Fig. 22).

The material from each of these three peaks was pooled, dialysed, and freeze dried as three separate samples.

Fractionation of LS/S.a./G100/DE-2 on UltrogelAcA34.

Further fractionation by gel filtration of the middle peak (0.6M salt) from the ion exchange column was carried out using Ultrogel AcA34 gels on columns 85cm x 2.6 cm using conditions as described in the Experimental Section. A calibration of the column using proteins of known size is shown ( Fig. 24).

The glycopeptide samples (120 mg) were applied in 5.0 ml of the pH 7.5 eluting buffer and eluted in an ascending manner.

Fractions of 5.0 ml were collected, and analysed for carbohydrate using the phenol/$H_2SO_4$ method. The results (Fig25) indicated that the material was still polydisperse after all the steps of purification the material had undergone. Recovery of the material applied was 83% , and the eluate was arbitrarily divided in two equal fractions as indicated (Fig.25).

TABLE 12

Analysis of Purified Fractions ( u moles / mg )

| Sample | * Hexosamine | * Hexose | * Sulphate | Sulphate Hexosamine |
|---|---|---|---|---|
| L.S./S.a. G100 | 1·31 | 1·70 | 1·52 | 1·16 |
| L.S./S.a. G100 DE 1 | 0·75 | 0·83 | 1·26 | 1·68 |
| L.S./S.a. G100 DE 2 | 1·15 | 1·45 | 1·88 | 1·63 |
| L.S./S.a. G100 DE 2 /A34-1 | 0·92 | 1·34 | 1·43 | 1·55 |
| L.S./S.a. G100 DE 2 /A34-2 | 0·72 | 0·65 | 1·25 | 1·74 |
| L.S./S.a. G100 DE 3 | 0·61 | 0·77 | 1·18 | 1·93 |
| Tope G150 | 1·08 | 1·51 | 0·19 | 0·17 |

* All the above results are expressed in u moles / mg.

## Studies on the Alkaline Degradation of Glycopeptide Preparations from Squalus acanthias and Galeorhinus galeus

### U.V. Absorbance

The absorbance in the ultra-violet at 241 n m of samples L.S./S.a./G100 and Tope G150 was found to undergo a dramatic rise after treatment with 0·5 M NaOH for 48 hours at 25°C. :-

Table 13 Effect of Alkali on absorbance at 241 n m of Lorenzan glycopeptides.

| | Before Alkali | After Alkali |
|---|---|---|
| Squalus acanthias | 0·29 | 2·1 |
| Galeorhinus galeus | 0·34 | 3·42 |

This tenfold rise in absorbance was quite repeatable and represents the values obtained for exactly equivalent amounts of material before and after and between the species. The effect in Galeorhinus galeus was consistently somewhat greater than that observed in Squalus acanthias.

Table 14 Effect of Alkaline Treatment on Elson-Morgan Chromogen Formation in the Reissig (1955) Assay

| | Absorbance at 585 n m | |
|---|---|---|
| | Untreated | Alkaline Treated |
| Squalus | ·004 | ·143 |
| Squalus (boiled) | ·046 | ·820 |
| Galeorhinus | ·000 | ·210 |
| Galeorhinus (boiled) | ·054 | 1·40 |

The results show a marked absorbance in the formation of direct (unboiled) chromogen after alkaline treatment; in parallel with the increase in absorbance at 241 n m., the effect on Galeorhinus galeus is

more pronounced than that on Squalus acanthias. The results for the indirect ( with boiling) chromogen formation show a marked rise in absorbance following alkaline treatment, but the increases are also much greater when we compare the indirect and direct chromogen formation following alkaline treatment.

## Effect of Alkaline Degradation on the Amino Sugar Analysis of the Glycopeptides

Table 15 shows the change in ratio and amounts of the two amino sugars following alkaline treatment. Galactosamine is seen to be substantially destroyed, in parallel with the destruction of threonine, whilst glucosamine remains unaffected.

## Effect of Alkaline Treatment on the Molecular Size of the Glycopeptides

The distribution of molecular sizes is markedly affected by the alkaline degradation treatment; the gel filtration experiments on Biogel P2 columns before and after alkaline degradation show that a real degradation in molecular size has occurred (Fig 28), and

TABLE 15.

Destruction of Galactosamine
Following Alkaline Degradation

(Figures expressed as a percentage of total Hexosamine. G.L.C. Analysis as Butyl Boronate Esters)

| | % Glucosamine | % Galactosamine |
|---|---|---|
| L.S./S.a. - G100 | 74.8 | 25.2 |
| L.S./S.a. - G100 - Alk. | 94.0 | 6.0 |
| Tope G150 | 53.8 | 46.2 |
| Tope G150-Alk | 87.8 | 12.2 |

Figures expressed as u moles/mg. :-

| | Glucosamine | Galactosamine |
|---|---|---|
| L.S./S.a. - G100 | .980 | .330 |
| L.S./S.a. - G100-Alk. | .980 | .062 |
| Tope G150 | .581 | .499 |
| Tope G150-Alk. | .588 | .082 |

multiple small molecular weight components replaced the (excluded) macromolecular material to a considerable extent, although some material remained in the excluded zone, even after alkaline degradation.

Amino Acid Analysis of the Lorenzan Sulphates - The Proteoglycans and the Glycosaminoglycans

Table 16 shows the results obtained for the amino acid analysis of the whole jelly from Squalus acanthias. Column (1) shows the results in n-moles/mg for Squalus. Since four of the amino acid residues had closely similar values, namely glycine, valine, leucine and lysine, the values of all amino acids obtained were divided by the average of these four. This value is 75·25. This in turn, gave for the lowest figure, a value of 0·21, and accordingly, to test for molar equivalence, all these values were multiplied by 5 to give column 3. Putting the lowest value $\frac{1}{2}$ cystine = 1·0 (actual value, 1·05), the final column shows the nearest integral value. Most amino acid values after this procedure lie fairly close to an integral value which may indicate that the results are not inconsistent with the presence of but one protein species. All that can really be claimed is that it is convenient to look at the results in terms of simple molar ratios. The analysis figures for this particular sample derive from the Locarte analyser before its updating to automatic sample application, different column packing, and automatic calculation of the results. The resolutions are, therefore, somewhat different from those obtained in all subsequent runs. The significance of this is that contamination of some amino acids with the large amounts of hexosamine is different.

Tyrosine and phenylalanine values are not quoted, as they were found

to be coincident with the elution times for glucosamine and galactosamine respectively. Destruction of the amino sugars by the strong hydrolysis conditions was observed in model runs, and was so great as to render hexosamine analysis impossible this way.

% Loss on Hydrolysis of Amino Sugars

| | |
|---|---|
| Glucosamine | 53·7 % |
| Galactosamine | 62·4 % |

The significant finding is the large amount of threonine relative to all other amino acids, whilst glutamic acid is present in substantial amounts.

Table 17 shows the amino acid analysis results for the native jelly of Galeorhinus galeus. Threonine is seen to be again easily the most abundant amino acid, followed by glutamic acid.

Table 16

Sample of Original Jelly from Squalus acanthias for Amino Acid Analysis (Dialysed, Freeze-dried)

| | Col. 1 | Col. 2 | Col. 3 | Col. 4 |
|---|---|---|---|---|
| | n.moles/mg. | Col. 1/75.25 | Col. 2 x 5 | Nearest Integer |
| Asp | 111·5 | 1·48 | 7·4 | 7 |
| Thr | 283·0 | 3·76 | 18·8 | 19 |
| Ser | 103·5 | 1·38 | 6·9 | 7 |
| Glu | 179·5 | 2·38 | 11·9 | 12 |
| Pro | 137·0 | 1·82 | 9·1 | 9 |
| Gly | 74·5 | 0·99 | 4·95 | 5 |
| Ala | 149·0 | 1·98 | 9·9 | 10 |
| ½ Cys | 15·5 | 0·21 | 1·05 | 1 |
| Val | 73·0 | 0·97 | 4·85 | 5 |
| Met | 26·0 | 0·35 | 1·75 | 2 |
| Ile | 117·5 | 1·56 | 7·8 | 8 |
| Leu | 74·5 | 0·99 | 4·95 | 5 |
| His | 27·5 | 0·37 | 1·85 | 2 |
| Lys | 79·0 | 1·04 | 5·20 | 5 |
| Arg | 116·0 | 1·54 | 7·7 | 8 |

Column 2 represents values obtained when results are divided by the average of the four most closely agreeing figures.

Based on the assumption of a single protein species, the minimum molecular weight works out for this material at 13,130, which figure takes no account of tyrosine or phenylalanine.

TABLE 17

Lorenzini Jelly from Galeorhinus galeus

Amino Acid Analysis (Dialysed, Freeze-dried)

| | n. mole/mg x 10 | ÷ 11·8 | Nearest Integer |
|---|---|---|---|
| Asp | 62·7 | 5·3 | 5 |
| Thr | 235·5 | 19·95 | 20 |
| Ser | 62·7 | 5·3 | 5 |
| Glu | 68·3 | 5·79 | 6 |
| Pro | 57·8 | 4·89 | 5 |
| Gly | 50·2 | 4·25 | 4 |
| Ala | 73·9 | 6·26 | 6 |
| Half Cystine | | | |
| Val | 50·8 | 4·30 | 4 |
| Met | 11·8 | 1·0 | 1 |
| Ile | 86·4 | 7·32 | 7 |
| Leu | 35·5 | 3·00 | 3 |
| Lys | Not done | | |
| His | " | | |
| Arg | " | | |

TABLE 18

Lorenzini Glycopeptides : Partial Amino Analysis

Before and After Alkaline Degradation

Results are expressed in n moles/mg.

| Sample | L.S./S.a.G100 | L.S./S.a.G100/Alk | TopeG150 | Tope G150 Alk |
|---|---|---|---|---|
| Asp | 23·60 | 23·60 | 16·29 | 26·61 |
| Thr | 36·22 | 30·16 | 116·45 | 27·15 |
| Ser | 13·21 | 13·22 | 6·21 | 6·13 |
| Glu | 43·00 | 43·06 | 27·96 | 28·91 |

Formation of α-aminobutyric acid on borohydride reduction, followed by alkaline degradation

| Sample | L.S./S.a./G100/$BH_4$/ Alk. | Tope G150/$BH_4$/ Alk. |
|---|---|---|
| α - A.butyrate | 2·71 | 3·54 |

Effect of Alkali on the Amino Acid Composition of the Glycopeptides

Table 18 shows the partial amino acid analysis of the glycopeptides from the two species under consideration before and after alkaline treatment, and also after borohydride reduction following alkaline degradation. The outstanding point of interest, however, is the marked destruction of threonine which follows alkaline degradation, and which occurs in both species, and the formation of α-amino butyric acid on reductive alkaline degradation.

## Lorenzini glycopeptides from a range of different animals

Table 3* presents analytical results obtained for analysis performed on crude jelly from a range of sixteen animals, the last two of which, Hydrolagus affinis and Chimera monstrosa belong to the group of holocephalic animals, and are distinguished from the others which are elasmobranch in nature.

Nitrogen results show a fairly wide range of values, Cetorhinus maximus, the basking shark, having a very high nitrogen value , probably reflecting the comparative 'stiffness' or rigidity of the gel found. All material was metachromatic save that from the tope, Galeorhinus galeus, which is in accord with other analytical information on this particular material.

A wide variation between the species is found in the amino sugar ratios, so that in Raja naevus, there is a glucosamine to galactosamine ratio of 7·06, while at the other end of the range, in Hydrolagus affinis, there is a ratio of 0·126. Fig. 26 shows these results plotted graphically for three animals.

The values obtained for uronic acids show some variation, but are uniformly very low in relationship to the other analytical figures. These values, together with the difficulty found in identifying uronic acid in hydrolysates chromatographically, suggests that there really is no such substance present. The small amounts apparently found in the colorimetric analysis might be accounted for by the interference shown by galactose in the assay (Fig. 27 ), a fact not mentioned in the original paper by Bitter & Muir (1962).

* see Page 44a

## Ageing Changes in Proteoglycan Distribution in Cartilage compared with the Situation in Mammals

Table 19 summarises the analytical results for pectoral girdle cartilage from Squalus acanthias expressed as a function of age as represented by the length (and weight) of the animals. The length of females is said to be greater than that of males of comparable age, an observation made by Holden & Meadows (1962).

However, the results of main interest here relate to what happens to the proteoglycans as the animal ages. The total hexosamine content is roughly the same as the total uronic acid content. Since the uronic acid values are likely to be somewhat high, and the total hexosamine values somewhat low as a consequence of hydrolytic destruction, it is possible that the actual hexosamine values are rather higher than the uronic acid values.

Both sets of values are seen to drop off in concentration ( in the dried cartilage samples used) quite markedly with age. In part, this is due to the enormous increase in the inorganic content of the cartilage which approaches nearly 50% of the dry weight of the cartilage in the older animals, compared with the situation in the youngest animals examined in which inorganic matter is between 10 - 20% of the dry weight. Indeed , the inorganic matter plotted as a function of length (Fig 30) shows a linear relationship with age, but with a different slope for the two sexes. The regression coefficient for males worked out at 0.63, and for females, 0.44. (Correlation coefficients are 0.90 and 0.93 respectively).

These differences between the sexes in the chemical analytical results of cartilage are consistent with the results of Holden & Meadows (1962) mentioned above.

| | Sex | Length (cm) | Weight (g) | Dry wt. uronic acid (μg/mg) | Dry wt. total hexosamine (μg/mg) | Total hexosamine glucosamine (%) | Total hexosamine galactosamine (%) | Dry wt. ash (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | ♂ | 27·9 | 63·0 | 85·6 | 99·0 | 9·9 | 90·1 | — |
| 2 | ♂ | 28·7 | 84·0 | 74·6 | 79·6 | 9·6 | 90·4 | 11·8 |
| 3 | ♂ | 31·9 | 99·5 | 74·0 | 82·3 | 11·1 | 88·9 | 19·9 |
| 4 | ♀ | 32·6 | 139·9 | 81·7 | 87·5 | 8·4 | 91·6 | 19·2 |
| 5 | ♀ | 36·7 | 163·0 | 85·0 | 80·6 | 8·2 | 91·8 | 16·6 |
| 6 | ♂ | 38·6 | 195·7 | 75·6 | 76·5 | 7·8 | 92·2 | 21·3 |
| 7 | ♂ | 40·1 | 193·9 | 80·5 | 80·5 | 10·0 | 90·0 | 22·8 |
| 8 | ♂ | 40·5 | 220·1 | 82·6 | 79·5 | 10·1 | 89·9 | 28·6 |
| 9 | ♀ | 45·0 | 348·6 | 79·1 | 75·5 | 6·1 | 93·9 | 19·3 |
| 10 | ♂ | 45·5 | 404·4 | 84·3 | 78·0 | 7·5 | 92·5 | 29·6 |
| 11 | ♂ | 45·5 | 352·6 | 78·0 | 73·6 | 6·4 | 92·6 | 22·6 |
| 12 | ♀ | 47·5 | 423·6 | 85·0 | 79·6 | 6·8 | 93·2 | 19·8 |
| 13 | ♂ | 48·5 | 416·3 | 83·6 | 75·1 | 9·1 | 90·9 | 27·6 |
| 14 | ♀ | 50·0 | 451·4 | 82·4 | 77·8 | 7·2 | 92·8 | 26·1 |
| 15 | ♂ | 51·5 | 462·0 | 92·1 | 84·7 | 7·5 | 92·5 | 23·1 |
| 16 | ♀ | 51·5 | 544·4 | 89·4 | 86·3 | 9·8 | 90·2 | 25·4 |
| 17 | ♀ | 53·5 | 547·9 | 82·8 | 81·1 | 8·0 | 92·0 | 23·6 |
| 18 | ♀ | 54·5 | 633·6 | 87·8 | 85·0 | 8·3 | 91·7 | 23·4 |
| 19 | ♀ | 58·0 | 716·8 | 84·4 | 83·9 | 7·4 | 92·6 | 24·0 |
| 20 | ♀ | 58·0 | 675·9 | 80·6 | 77·6 | 8·1 | 91·9 | 26·6 |
| 21 | ♀ | 58·0 | 647·8 | 81·2 | 78·0 | 7·5 | 92·5 | 28·0 |
| 22 | ♂ | 58·0 | 637·8 | 87·8 | 85·0 | 9·1 | 90·9 | 26·1 |
| 23 | ♀ | 58·5 | 734·8 | 81·7 | 75·5 | 8·6 | 91·4 | 25·0 |
| 24 | ♀ | 60·5 | 767·7 | 82·6 | 77·0 | 8·6 | 91·4 | 29·5 |
| 25 | ♀ | 61·5 | 811·9 | 83·4 | 77·2 | 7·1 | 92·9 | 22·5 |
| 26 | ♀ | 62·5 | 860·4 | 79·1 | 74·8 | 6·2 | 93·8 | 29·1 |
| 27 | ♀ | 63·5 | 954·6 | 85·3 | 75·0 | 8·2 | 91·8 | 28·8 |
| 28 | ♀ | 66·0 | 1044·7 | 90·6 | 83·0 | 6·5 | 93·5 | 29·1 |
| 29 | ♂ | 66·0 | 1008·0 | 54·4 | 56·8 | 8·7 | 91·3 | 35·7 |
| 30 | ♀ | 66·5 | 1112·4 | 82·3 | 77·5 | 10·4 | 89·6 | 36·0 |
| 31 | ♂ | 68·0 | 1129·0 | 28·2 | 29·0 | 10·9 | 89·1 | 48·6 |
| 32 | ♀ | 70·0 | 1318·6 | 81·4 | 75·0 | 9·5 | 90·5 | 33·4 |
| 33 | ♀ | 70·0 | 1420·8 | 83·7 | 73·8 | 6·6 | 93·4 | 37·0 |
| 34 | ♂ | 71·7 | 1267·0 | 63·8 | 54·5 | 6·9 | 93·1 | 48·6 |
| 35 | ♀ | 72·5 | 1358·0 | 76·2 | 70·0 | 9·0 | 91·0 | 38·1 |
| 36 | ♂ | 74·0 | 1680·0 | 38·4 | 38·9 | 9·1 | 90·9 | 39·6 |
| 37 | ♀ | 75·0 | 1820·0 | 78·7 | 76·5 | 6·3 | 93·7 | 34·1 |
| 38 | ♂ | 77·5 | 1628·0 | 54·4 | 57·1 | 6·1 | 93·9 | 43·0 |
| 39 | ♀ | 87·5 | 2531·0 | 61·2 | 58·0 | 7·5 | 92·5 | 34·3 |
| 40 | ♀ | 87·5 | 2540·0 | 80·0 | 81·0 | 10·7 | 89·3 | 38·5 |
| 41 | ♀ | 97·0 | 4660·0 | 48·9 | 96·6 | 10·2 | 89·8 | 47·5 |
| 42 | ♀ | 98·0 | 4545·0 | 39·4 | 53·0 | 11·2 | 88·8 | 44·0 |
| 43 | ♀ | 98·0 | 4320·0 | 17·5 | 21·0 | 11·2 | 88·8 | 43·7 |

Table 19 Analytical Results of Amino Sugar, Uronic Acid and Ash Content of Pectoral Girdle Cartilage from Squalus acanthias.

TABLE 20 - CHEMICAL ANALYSIS OF SAMPLES OF NASAL SEPTUM CARTILAGE OF THE HORSE

NASAL SEPTUM CARTILAGE : YOUNG & OLD (HORSE)

| ANIMAL NO. | LAYER | % TOTAL HEXOSAMINE | | % DRY WT. SIALIC ACID |
|---|---|---|---|---|
| | | GLUCOSAMINE | GALACTOSAMINE | |
| Young | I | 8.2 | 91.8 | 0.33 |
| 1 | II | 6.9 | 93.1 | 0.57 |
| | I | 12.2 | 87.8 | 0.38 |
| 2 | IIa | 10.8 | 89.2 | 0.63 |
| | IIb | 10.5 | 89.5 | 0.81 |
| | I | 11.9 | 88.1 | 0.30 |
| 3 | IIa | 9.9 | 90.8 | 0.43 |
| | IIb | 10.5 | 89.5 | 0.61 |
| Old | I | 15.7 | 84.3 | 0.46 |
| 4 | II | 24.8 | 75.2 | 0.85 |
| | III | 26.6 | 73.4 | 0.96 |
| | I | 20.2 | 79.8 | 0.52 |
| 5 | II | 25.8 | 74.2 | 0.82 |
| | III | 28.5 | 71.5 | 0.95 |
| | I | 21.2 | 78.8 | 0.44 |
| 6 | II | 29.1 | 70.9 | 0.90 |
| | III | 32.8 | 67.2 | 1.04 |

Whilst the total proteoglycan material is falling off with age in the dry material, the proportion of keratan sulphate, as revealed by the proportion of glucosamine does not change with age in any clear manner, and shows only expected biological variation around a value of 9%. In the wet cartilage, when allowance is made for the increasingly inorganic nature of the tissue, it is possible that this change is quite small, as connective tissue in common with other tissues, becomes progressively drier with age.

A number of measurements of sialic acid were made, but the values found were too low to be represented with confidence, and whilst trace amounts were found, the values showed no changes of a systematic nature.

That this behaviour of the proteoglycans of cartilage is peculiar to these animals can be seen in comparison with results obtained for horse nasal septum cartilage ( and in other mammals by other workers, considered in the Discussion Chapter.)

Table 20 shows the results of analyses of glucosamine and galactosamine for material from a series of six animals of increasing age varying from a foal (Animal I ) to advanced aged animal (Animal VI ). No precise ageing is available, but the animals were aged by a veterinary surgeon at the slaughterhouse. The ageing pattern of mammalian cartilage shows characteristic bands when stained by metachromatic dyes (Szirmai & Doyle (1959), Szirmai (1963) ). In the youngest animals, two zones are present, and as the animal ages, three zones of distinct levels of dyebinding are found. Analysis of material from these dissected layers is shown here as well as from the individual animals. The material in the centre shows ageing and dying cells whilst the

peripheral material shows younger and more vigorous cells to be present. A diagram of this change is shown in Fig. 31 by way of explanation.

As the animal ages, the amount of glucosamine as a percentage of the total hexosamine is seen to rise dramatically to a value around four times that of the youngest animal. Moreover, the "ageing" of the tissue shows the same sort of changes in the layers of 'aged' cartilage found within an individual animal as defined by Szirmai & Doyle (1959), and Szirmai (1963). Thus we see a larger proportion of glucosamine as we move towards the central 'older' layers of cartilage.

The variation in concentration of sialic acid in the tissue fractions appears to run roughly parallel to the glucosamine levels. As considered later in the Discussion Section, this probably reflects the association of sialic acid with the hybrid keratan sulphate molecular systems found in mammalian cartilage. This aspect of the differences between the two types of cartilage, that of elasmobranchs on the one hand, and that of mammals on the other, is further found in the uv-absorption curves found subsequent to the various treatments (Figs. 32 a-e ) outlined.

In Fig. 32a, we find after autoclaving of mammalian cartilage homogenates a plateau region from 260 - 280 nm. Autoclaving gelatinises the collagen and renders the turbid suspension a clear solution. Digestion of this with collagenase followed by dialysis merely lowers the general level of absorption. In Fig. 32b, when pepsin/trypsin was substituted for collagenase, involving digestion alternately at pH2·0 and at pH 7·5, we find two small additional peaks

at 260 nm and 269 nm. Fig. 32c shows that treatment of the collagenase material of Fig. 32a at pH 6·0 and 56°C for two hours, produced uv-absorbing peaks similar to those of Fig. 32b. Fig. 32d shows the collagenase-treated, and pepsin-trypsin treated material plotted on the same graph. Fig. 32e shows the results of treating elasmobranch cartilage by autoclaving, followed by collagenase treatment, and the same two hour heating period at 56°C and pH 6·0. In the elasmobranch cartilage, the uv-absorbing peaks are not produced by the treatment which manifested them when mammalian cartilage was used.

# CHAPTER FOUR

## DISCUSSION SECTION

### Methodology

Some aspects of the methods used in this thesis require comment.

### Proteolytic Digestion

In the early days of investigations into proteoglycans, or mucopolysaccharides, fairly strong methods were used to remove protein, including alkaline extraction, which is now known to cause substantial degradation of the glycosaminoglycan (carbohydrate) portion. Many workers considered the protein to be a contaminant, adventitious in nature, and a nuisance. However Ogston & Stanier (1952) studying hyaluronic acid, found that ultrafiltration produced a 'complex' with about 25% protein, when mild extractants were used in the preparation. They argued that this represented a true complex by virtue of its constant composition and the fact that any treatment except this ultrafiltration destroyed a most important and characteristic property - the non-Newtonian viscosity.

In 1954, Shatton and Schubert found that aqueous extraction of cartilage produced a product with compound-like properties,and they prepared fractions by alcohol precipitation which had a constancy of composition in terms of protein : polysaccharide ratio. In 1958, Mathews and Lozaityte published an analysis of chondromucoprotein. Partridge et al (1961) extracted the complex from cartilage with neutral salt solutions and published the amino acid analysis.

Following this, the reality of protein complexes has been greatly studied, and shown to be a widespread phenomenon in animal polysaccharide structure.

Thus, removal of the protein in order to study the polysaccharide part (including the linkage region) requires the use of mild proteolytic techniques, and this in turn means proteolytic digestion.

Pepsin and trypsin have been used, but the former requires fairly extreme acid conditions, and trypsin alone is of limited effectiveness. Scott (1960) popularised the use of papain which is an extremely heat-stable enzyme operating efficiently at 65°C. Material prepared in this study used this method. The method is open to the objection that oxidative-reductive degradation of the polysaccharide might occur in consequence of the requirements for reducing agents (cysteine) to be present.

Pronase B in pH 7·7 buffer in 10 m M $CaCl_2$ followed by 24 hour incubation at 37°C was tried, and proved at least as effective as papain in removing protein, and involving the mildest procedure. The product obtained was not noticeably different from that prepared using papain. This then became the method of choice, and all the material used for the alkaline degradation investigations was prepared in this manner.

Metachromasia

Dyes of the thionin group exhibit this useful property which involves binding of the positively charged dye to the polyanion.

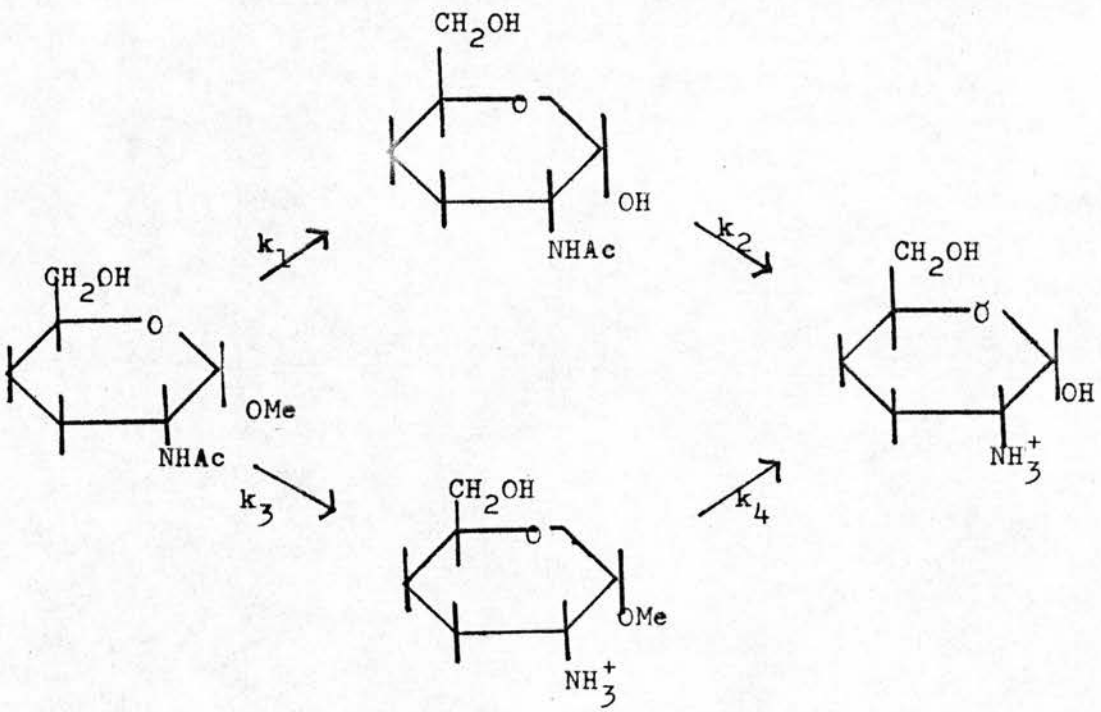
If the charge density is sufficient on the polysaccharide, and particularly if a repeating charged function exists, then the result is electronic perturbation in the dye-complex system, with a resulting shift in the colour of the dye. This binding can be made quantitative, and used to measure the degree of charged functions, and can also be used in dye-binding studies to titrate the acidic functions (Szirmai & Balazs, 1958). Thus, if the material or section is stained at pH2·0, only sulphate esters will be ionised, and metachromasia becomes an index of sulphated polymers. In tissue sections however, binding might be suppressed to an extent by the presence of protein which could acquire a cationic charge and, hence compete with the dye for the polyanion sites.

In the Lorenzini jelly studies reported here, metachromasia was studied at pH2·0, and hence the bathochromatic shift implies that only sulphate ester linkages are involved.

## Hexosamine Analysis

The problem of hexosamine analysis is a most intractable one, as destruction of the amino sugars is an important but variable factor. Moggridge and Neuberger (1938) considered the two paths possible for hydrolysis of 2- acetamido -2- deoxyhexose glycosides.

Pathway A




Pathway B

The values of the rate constants $k_1$, $k_2$, and $k_3$, proved to be roughly similar, but that for $k_4$ was of much lower value.

If deacetylation happens first, a free amino group results which is protonated under the acid conditions. In consequence, protonation of the glycosidic oxygen, prior to hydrolysis, becomes more difficult due to repulsive forces, thus causing a resistance of the glycosidic bond to hydrolysis.

Fig. 33 shows the results of an empirical hydrolytic study of the Lorenzini glycopeptide using 2 N HCl and 4N HCl. This enabled selection of conditions, in this instance, for the maximum release of hexosamine, but it is clearly not ideal, and is time-consuming. Strictly speaking, all such analyses should have their hydrolysis conditions established first, not always a practicable proposition.

Methanolysis rather than aqueous acid hydrolysis appears to result in little destruction of sugars, and this has been used to advantage by Chambers and Clamp (1971) for sugar analysis, including amino sugars, by gas-liquid chromatography . Because of the anomerisation and ring isomerisation involved, multiple peaks for each component sugar are produced, making interpretation difficult except for the most experienced.

One hydrolytic procedure used for quantitative release of sugars involves using a cation - exchange resin in the hydrogen form, together with a catalytic amount of mineral acid. Amino sugars, however, are bound to the resin and are not subsequently eluted in a quantitative manner for subsequent g.l.c. analysis (Niedermeier, 1971).

Porter (1975) used nitrous acid deamination of the resin bound hexosamine to give neutral 2,5-anhydrohexoses, and chromatographed these as their corresponding neutral alditol acetates. Glucosamine would give 2,5-anhydromannose, while galactosamine would result in 2,5 anhydrotalose, both of the D-configuration, using this method.

Porter found a linear relationship between detector response and the concentrations of glucosamine and galactosamine used in model experiments.

This recent method has not been used in this study, but it would appear to offer the possibility of improving on the existing systems for amino sugar analysis, excepting for the rather complex methanolysis methods.

The Organs of Lorenzini

The plates of the sagittal sections through the snout (Plate 1) show that the organs of Lorenzini are fairly large sense organs, and it would seem unreasonable to suppose that they are other than important to the animal for this, if no other, reason. Elasmobranchs have no swim bladder, and a dead one floats on the sea, which accounts for the material from the basking shark, Cetorhinus maximus, becoming available for this study.* This means, of course, that all such animals are required, like the Flying Dutchman of legend, to voyage continuously. Only by moving can they control their depth, and their oxygen supply as well, and this in turn means that enormous distances must be travelled, even in the smaller species in a genus conspicuous in general for the largeness of the species within it. Thus, the requirement for a navigational system becomes apparent.

For a long time, the organs of Lorenzini were considered by many physiologists to be a part of the lateral line organ with which indeed they share a common embryological origin. This lateral line organ can detect vibrations at considerable distances, and together with the animal's acute sense of smell, enables it to find prey effectively, Whilst the Dutch group have established that elasmobranchs can detect the spread muscle potentials of other animals,

these potentials fall off

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* Many elasmobranchs have negative buoyancy, i.e., they sink, but the problem remains the same, they must swim to maintain any given depth.

as a power function of distance, and hence can only be used to detect animals at very short range.

Murray (1959) studied the effects of temperature and salinity changes, and of weak direct electric currents. A more detailed investigation of an electrophysiological kind was made by him later at Woods Hole (Murray, 1965). In these studies, Murray found that there was a response to electrical impulses, and he considered the possibility that the response to temperature and to salinity changes might reflect changes in junction potentials produced. This does not mean, however, that these responses are necessarily adventitious; in much of the territory covered by many of these species, thermoclines exist, in which rapid changes in temperature and salinity occur, and with them, the distribution of other animals.

The organs respond sensitively to a large variety of stimuli, pressure, temperature, pH, salinity and electric potential changes, and whilst all of these may be sensed by some species to their advantage, the evidence for the behaviour of the organ as a compass, or a detector of magnetic fields seems intriguing, and perhaps most convincing. That the organ is capable of responding to many different stimuli, does not mean that the animal cannot discriminate between the various sorts of stimuli, and this view would give enormous importance to the existence of such an organ.

The Nature of the Jelly

Solubilised material shows marked metachromatic activity at pH2·0 which can only result from sulphuric acid ester residues, as no other function is likely to be ionised at this pH, and thus bind cationic dyes.

The dialysed, freeze-dried material showed this activity, as might be expected, as well as the native jelly, thus confirming that metachromatic activity derives from macromolecular material.

Whilst uv. spectral analysis does not show the characteristic protein band around 280 nm., the analytical figures for nitrogen suggested that protein was present in the crude dialysed material. Some of the carbohydrate material absorbing at the lower end of the uv presumably is serving to mask the appearance of the protein band.

If Jensen's (1956) observations based upon enzymatic degradation were correct, we should expect to find, from the chondroitin sulphate, N-acetyl-D-galactosamine in equimolar proportions with D-glucuronic acid and from hyaluronic acid, N-acetyl-D-glucosamine also in equimolar proportion with D-glucuronic acid. Whilst paper chromatography and, subsequently, gas-liquid chromatography showed that both hexosamines were present in hydrolysates, D-glucuronic acid was not found, despite much searching, and this observation was supported by the evidence from quantitative analysis by the Bitter & Muir (1962) method. Using controls containing the same amount of galactose as that found to be present, the small amounts of uronic acid apparently present according to this method could be accounted for on the basis of no uronic acid whatever, although the absorption spectra of the chromogens was different (Fig. 27).

That galactose is the monosaccharide responsible for the various neutral hexose reactions - Molisch reaction, anthrone reaction, phenol/sulphuric reaction is seen by the paper chromatographic and GLC analysis, and confirmed by the formation of a derivative. Behaviour of the material towards D-galactose oxidase establishes this hexose as the D-isomer.

Papain digestion of the jelly from Squalus acanthias produced a substance which proved to be a glycopeptide which contained roughly equimolar proportions of galactose, total hexosamine, acetyl groups (which from their behaviour in exposing ninhydrin-reacting groups on hydrolysis, were N-acetyl groups), and sulphate. These analytical figures for the crude preparation certainly rule out the supposed mixture of chondroitin sulphate and hyaluronic acid; indeed, they are sufficient to rule out the presence of either of these components in any mixture contemplated.

Electrophoretic Results

The results of the paper electrophoresis (Fig. 20 ) obtained at this stage in the investigation were crucial to understanding the nature of the material. Although the tentative conclusions proved to be substantially correct, at this stage they were made without any appreciation of the high level of heterogeneity obtaining which emerged later in the study. Since the separations were performed on both native and on crudely purified material from proteolytic digestion, it was possible to see that the relatively protein-free material moved towards the anode at about half the rate of chondroitin sulphuric acid (a mixture of the four sulphate and the six sulphate isomers).

Thus, at this stage in the investigation, the material appeared to be a sulphated glycosaminoglycan with D-galactose, ester sulphate, N-acetyl aminosugars of both types, and no uronic acid. The nearest known substance to this was keratan sulphate, but the presence of both amino sugars in this degree did not accord with this entirely. The hexosamine analysis on the material pooled after electrophoretic separation showed similar ratios of the hexosamines. In the case of

Squalus acanthias, this was close to 4:1 for glucosamine: galactosamine.

Hyaluronidase Treatment of the Glycopeptide

Hyaluronidase of testicular origin is capable of breaking down a range of glycosaminoglycans, including hyaluronic acid and chondroitin-4-sulphate and chondroitin-6-sulphate. In this study, it appears to be without effect on the Lorenzini glycopeptide, in as much as gel filtration indicates that the material is still macromolecular at the end of prolonged incubation. The material eluted from Sephadex G75 shows some slight (but repeatable) variation in the hexosamine ratio, the larger molecular weight material showing a decreased glucosamine content with respect to galactosamine. This difference was sufficient to raise the question as to the homogeneity of the Squalus acanthias glycopeptide material, and to prompt the further investigations into this aspect. These preliminary slight indications of heterogeneity seemed at variance with the results of glucosamine/galactosamine ratios previously observed.

Further Purification of the Squalus Glycopeptide

Figure 21 shows that in the DEAE-Sephadex analytical column used with a stepwise salt gradient, the material is distinctly heterogeneous. Because of the eluting medium used - an increasing salt concentration - the tentative conclusion is that there is a degree of heterogeneity with respect to charge in the system. As the answers to such questions must finally reside in analytical results, the DEAE-Sephadex column was scaled up to obtain sufficiently large amounts of material for this purpose and for further fractionation. (Fig 22).

The recovery of material in this procedure is good, 800 mg in toto, representing 94% of the material applied. More than half of this is found in peak 11, yet analysis shows that, with respect to either hexosamine, this is the least sulphated material (Table 21 ). However, especially as found in the material eluted at 1.2 M salt (Peak 111), there do exist marked differences in the degree of sulphation between the fractions.

The main fraction from the DEAE-Sephadex separations, L.S./S.a. G100 DE-2, shows that this material is still polydisperse with respect to size, but on the Ac[illegible]4 molecular sieve column used, the cut made for analytical purposes was quite arbitrary and not based upon distinct resolution. This appears to reflect the real situation and was not, for example, a consequence of overloading the column, for comparable results were found on reducing the sample applied from 100 mg. to 25 mg.

Purification of Galeorhinus galeus Glycopeptide

The Sephadex G150 columns used following either papain or pronase digestion (no great differences were found in the resulting products of digestion) showed a main carbohydrate-protein fraction, followed by a tail of smaller molecular weight material contaminated by adventitious protein. Fig. 34 shows the analysis of fractions for carbohydrate and protein of proteolytic digests of Tope material. Gel filtration columns (Sephadex G-150) were used. The fractions numbered 6-13 inclusive were collected and pooled, and no further fractionation of this

main fraction attempted. It is either not sulphated, or at least not greatly so, for although the analytical figures ( Table 12 ) indicate extremely small amounts of sulphate do seem to exist, and the I.R. evidence shows some degree of absorption in the sulphate bands, the material does not exhibit any metachromasia, although it may well be binding small amounts of dye.

## Analytical Results on the Purified Fractions

Table 12 shows the results for the purified fractions in terms of hexosamine, hexose and sulphate. Hexosamine values, and hexose and sulphate levels also, appear to decrease as fractionation proceeds. As judged particularly by the results of the Ultragel AcA34 chromatography, the larger molecular weight material is more sulphated in the case of Squalus acanthias. The general lowering of the carbohydrate levels probably indicates that more non-carbohydrate material (protein) is associated with these fragments. The results of the carbohydrate and sulphate analysis indicate a high degree of polydispersity between the fractions. In the case of Galeorhinus galeus , since no further fractionation was performed, we have a "deproteinised" component extremely low in sulphate, but not, from these figures, entirely without it.

## Amino Acid Analysis of the Jelly Material

Table 16 gives the results in terms of integral molar ratios, of the analysis for Squalus acanthias, while Table 17 gives that for Galeorhinus galeus.

The method used in calculating the former may seem rather more complex than is required, but the striking closeness of analysis of four of the acids prompted use of their average as a basis for comparison and gave a high degree of confidence. No such picture emerged in the analysis of tope jelly, and the results obtained here show much greater deviations from integral ratios.

In both species, the dominant amino acid found in the undigested material is threonine. A major difference between the two species is the larger amounts of glutamic acid present in Squalus acanthias jelly.

As no results for the basic amino acids are available for Galeorhinus galeus, the comparison between the two cannot be made complete. For these amino acid results available, and this is the majority, there appears to be less protein generally in Galeorhinus galeus, and this is borne out by the nitrogen analysis of the material from both in Table 3, (Page 44a).

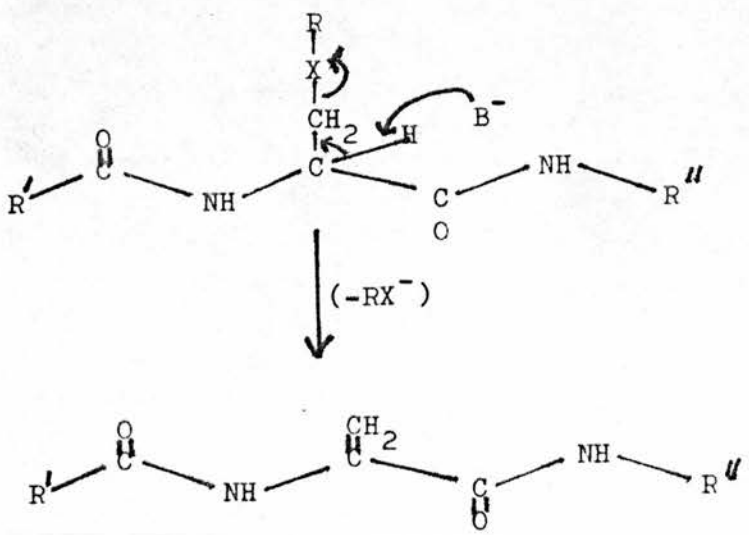
## Structural Studies on the Glycopeptides

### The Carbohydrate - Protein Linkage

Although a number of less common types of linkage between carbohydrate and protein have been described recently, two main types have been clearly established.

Type (1) involves the amide nitrogen of asparagine in the protein backbone linked to the $C_1$ of a hexosamine residue, first described by Fletcher et al (1963) and Grant and Simkin (1964). This N-acylglycosylamine bond is relatively stable to acid or alkali (Kent, 1967).

Type (2) linkage involves the side chain of serine or threonine glycosidically linked to a monosaccharide residue. Muir (1958) first drew attention to this type of bond when studying the results of proteolytic degradation of chondroitin-sulphate protein of cartilage. The alkali-sensitivity of the bond prompted Muir to suggest that something like an ester link, involving the hydroxyl group of serine, was involved. Lindahl and Roden (1965) (see introduction) showed that the linkage was the serine hydroxyl to the pentose, xylose, glycosidically. Ginsberg and Wilson (1964), studied the effect of alkali on O-substituted serine residues in synthetic peptides, and showed that $\beta$ -elimination of an O-substituent occurred :-



The alternative route forming oxazolines was not favoured if $R^1$ and $R^{11}$ were polypeptide because hydrogen bonding limited the conformation.

Anderson et al (1963, 1964) demonstrated that chondroitin-sulphate protein and keratan-sulphate protein, when treated with 0·5 M NaOH at 20°C lost serine and threonine respectively. Reaction with alkaline borohydride results in an increase in alanine, or in the appearance of α - aminobutyric acid, de novo, deriving from serine or threonine respectively. Amino acid analysis requires hydrolysis conditions which result in the destruction of the unsaturated acids formed during β -elimination, and hence the need for reduction in order to detect their presence.

In keratan sulphate, the linkage demonstrated by Bray et al, (1967) involved serine or threonine, and N-acetylgalactosamine. This type of linkage seems more common in glycoproteins than in proteoglycans. It occurs in blood-group substances (Kabat et al, (1965) ), and in bovine submaxillary mucin glycoprotein, Tanaka and Pigman (1965). The latter authors treated the glycoprotein in 0·3 M $NaBH_4$ in 0·1 M NaOH at 5°C for 216 hours. Hydrogenation followed, using $NaBH_4$ in the presence of palladium chloride. Under these conditions, the loss of 16·6 u moles of threonine resulted in the formation of 16·2 u moles of α -aminobutyric acid.

Bhavanandan (1964) treated ovine submaxillary glycoprotein with 0·5 M NaOH at 0°C for 20 hours and 68 hours. Serine was destroyed to 44 and 49·390, and threonine to 40 and 50·5% respectively. The combined losses were almost equivalent to the amount of free N-acetyl-galactosamine which appeared in the medium. During degradation, the absorbance at 241 n m rose steadily, pointing to the formation of α-amincacrylic acid derivatives.

Bray et al (1967) studied the conditions required for the reducing sugar exposed after the $\beta$ -elimination to give a Morgan-Elson chromogen. Under the alkaline conditions used, the chromogen is formed provided that the galactosamine residue is not substituted at $C_4$.

A better leaving group than OH is required at $C_3$, since free N-acetylhexosamines give little or no chromogen in 0·5 M NaOH at 25°C. Thus, a positive test in the Reissig assay modified to exclude the heating at 100°C for three minutes at pH9·0, (as described in the Chapter on 'Methods' ) provides evidence for this type of linkage. In the case of the Lorenzini glycopeptides, Table 20 shows that some destruction of threonine occurs in both Squalus material L.S./Sa.G100 and in Tope G150 preparations after alkali treatment.

In these purified digests, there is a considerable amount of threonine remaining after proteolytic digestion which gives the first clue to the existence of a threonine link. In the case of Squalus acanthias glycopeptide, although threonine is the predominant amino acid in the mative material, after digestion, glutamic acid appears to usurp this role, although threonine is the next amino acid in terms of quantitative importance. Alkaline treatment results in formation of a Morgan-Elson chromogen, and in some destruction of threonine. (Tables 14 and 18 ) This is paralleled by a marked increase in absorbance at 241 n m , and by the appearance of $\alpha$ - aminobutyric acid in the hydrolysates of reduced samples, although not in amounts equivalent to the amount of threonine destroyed. Apparently, more strenuous reducing conditions would be required to achieve this, for example, the use of palladium catalysts as described by Bhavan[illegible] (1964).

The Tope G150 material shows the same properties, except that it does so to a much greater degree; the observed destruction of threonine is quite dramatic. The formation of Morgan-Elson chromogen is also more marked, together with the rise in absorption at 241 n m and the eventual formation of α-aminobutyric acid. A possibly significant difference is that the glutamic acid concentration in Galeus galeorhinus material is not nearly so great after proteolytic digestion as it is in the case of Squalus acanthias, although it does remain fairly high. In neither species does alkaline degradation have any effect on the concentration of glutamic acid in the glycopeptide. The results of the separation of the alkali degraded material on Biogel $P_2$ tend to support the differences between the material from the two species (Fig. 28 ).

In Squalus glycopeptide, alkaline degradation affects the elution pattern only very slightly, implying that degradation is not as great as in Galeus glycopeptide similarly treated, where rather smaller molecular weight material is formed, as judged by its later elution. Calibration of the column with dextran blue, sucrose and glucose shows that in the case of both glycopeptides, the untreated material is excluded, but that the treated material is not degraded as far as monosaccharides to any great extent, certainly not in Squalus material. Qualitative analysis of direct Morgan-Elson chromogens indicated that the chromogen occurred largely in the middle region, implying that some of it was linked to oligosaccharide material.

If the original galactosamine residue was linked at $C_1$ to a threonine residue, ( and Table 15 shows that galactosamine is destroyed simultaneously with threonine) then the linkage must be via $C_3$, $C_4$ or $C_6$ of the amino sugar. A residue linked at $C_3$ would be

eliminated during chromogen formation, and a $C_4$ substituent would prevent its formation as established by Bray et al (1967). Thus the oligosaccharide chain can only be attached to the chromogen at $C_6$.

Since a requirement for chromogen formation under the conditions used is that $C_3$ is substituted - a better leaving group than OH was a requirement established by those workers, it follows that some of the galactosamine residues are linked at position $C_1$, $C_3$ and $C_6$. Thus we have a model for (at least some of) the alkaline degradation studies as follows :-

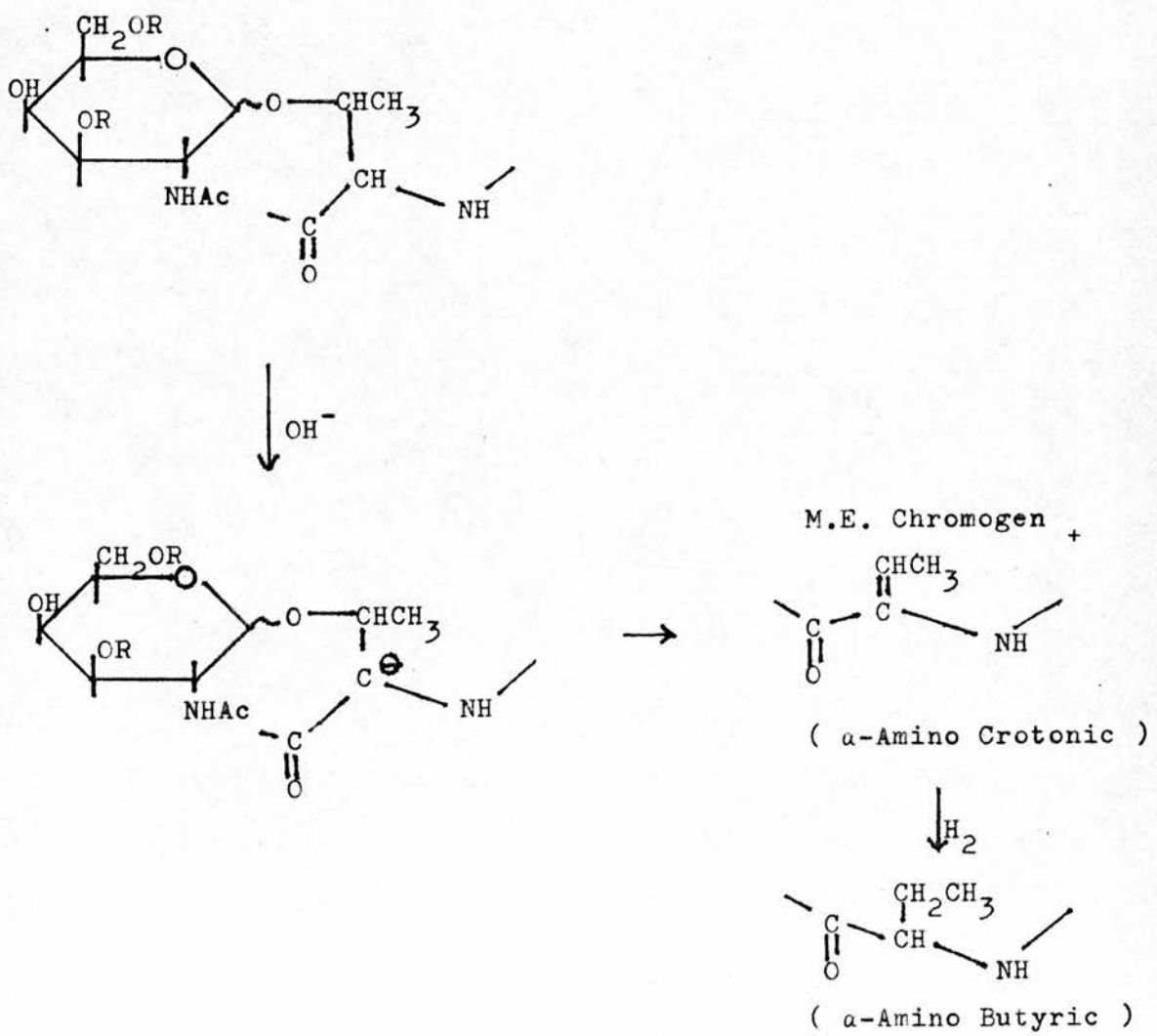
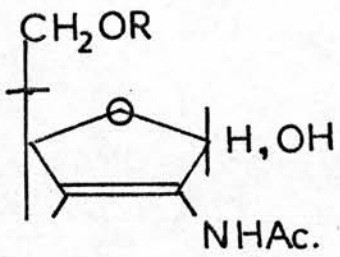


Table 14 shows that the Reissig assay for acetyl hexosamine is greatly increased after the more usual heating period at pH9.0. This means that not all hexosamine, indeed not all galactosamine, is involved in this type of linkage. It is equally true to say that not all of the threonine in the proteoglycan is involved in such linkages. The Morgan-Elson chromogen has a structure according to Kuhn and Kruger (1956, 1957) as follows :-




Hence the elimination of the $R^1$, in what was $C_3$ of the hexosamine, becomes apparent.

The relatively small degree of galactosamine - threonine linkage of Squalus acanthias glycopeptide compared with that found in Galeorhinus galeus (and in Cetorhinus maximus and Raja Clavata by How, Jones and Doyle (1969) ) together with the fairly large amounts of glutamic acid prompts the question of the possible involvement of this acidic amino acid in a further linkage between carbohydrate and protein. The Biogel $P_2$ evidence after alkaline treatment shows little signs of molecular break-up in Squalus glycopeptide. Hopwood and Robinson (1974) have produced evidence that skeletal

keratosulphate has an alkali-labile glycosidic bond between N-acetylgalactosamine and threonine. In addition, they postulate an alkali-stable bond between glutamic acid (or glutamine) and an unknown residue in the keratan sulphate structure.

It seems possible that this sort of structure exists in Squalus glycopeptide; indeed, it may exist in all the Lorenzan sulphates, but the indications are that if it does exist, then it does so to a greater degree in material from this species than in the others so far examined.

From the evidence of Hopwood and Robinson (1974), and also from the work in this study, it seems that keratan sulphates and their relatives contain bonds reminiscent of those in glycoproteins rather than in proteoglycans, and they also have branching in the carbohydrate moiety more characteristic of glycoproteins than of proteoglycans. On the other hand, the repeating sugar and sulphate functions known to occur in the keratan sulphates place them within the proteoglycan class as do some other properties. Thus it is possible to suggest that the keratan sulphate-like substances occupy an intermediate position in this classification system. The classification is none the less justified; the difficulty is that few of the criteria are absolute, the category depends upon the structures conforming generally to a number of properties rather than to any individual property exclusively.

## Comparative Studies on Lorenzini Jellies

Table 3* shows the basic analytical information obtained for a number of species, two of which are investigated in some further detail in this study, and two others have been further studied outside the matter of this thesis by How, Jones and Doyle (1969) and How, Jones and Stacey (1969).

These detailed studies establish the existence of a high degree of polydispersity within the "protein-free" carbohydrate derived from any of the single individual species. This variability is seen also between the species, where the analytical results presumably reflect the average for the mixture. Thus, we have variability in the hexosamine ratio and in the degree of sulphation between the average values for the individual species. Fucose was found to be present to a small extent in glycopeptides prepared from Cetorhinus maximus, the basking shark, (How, Jones and Doyle 1969), and small amounts were also observed in gas liquid chromatograms in Squalus acanthias in this study. As fucose has been known for some time to be a component of keratan sulphate, this is another point of identity between the two groups of substances. The publication of the composition of the material from Squalus acanthias, and its partial characterisation prompted the survey of a number of species, and resulted in the publications included at the end of this thesis. These are :-

(1) DOYLE J. Biochem J. 103, 325, 1967
The "Lorenzan Sulphates" : A New Group of Vertebrate Mucopolysaccharides.

(2) DOYLE J. Comp. Biochem. Physiol. 24, 479, 1968.
The "Lorenzan Sulphates" : A Comparative Study.

* see Page 44a

At this stage, the analytical information led to the group of compounds being named the 'Lorenzan sulphates', in line with their biological origin, and modern terminological practice. The variation found between the species in overall composition seems fairly large, greater, for example, than that found in most connective tissue proteoglycans between mammals. In the two holocephalic animals examined - these are cartilaginous fish, but not elasmobranchs - the difference seems very great indeed. The level of galactosamine found is vastly more than that found in any other keratan sulphate-like polymer. As no photograph is available, I include a line drawing of Hydrolagus affinis; Chimaera monstrosa is similar in appearance but rather smaller, and common enough in deep water off Iceland.

The very low (or possibly zero ? ) levels of sulphate ester found in the tope (Galeus galeorhinus) mean that this function is not required for the maintenance of a stable gel, which is intriguing, for many three dimensional gel networks involve the multiple charged functions of the glycosaminoglycans in their structure. No work has as yet been done on this aspect of structure. There is also no evidence for the presence of other protein or proteoglycan, such as hyaluronic acid, to form the large protein/proteoglycan constructions which are now known to exists in connective tissue (Heinegard, 1972, Heinegard and Hascall 1974, Heinegard and Axelsson 1977), and which must play an important role in such phenomena as fluid exchange and molecular exclusion.

The lack of sulphate ester in this species further means that this is not required for the electrical conductivity required by the physiological function of the organ. This presumably involves the movement of ions such as sodium throughout the gel network. At one

end, in most sea waters we have roughly 0·5 M NaCl, and just over half of that in the animal's body fluid. (In elasmobranchs, much of the osmolality of the body fluids is made up of urea, a consideration which puts constraints upon the sorts of protein structures permissible, and which does nothing useful for its culinary properties either).

Fresh water sharks do exist in Lake Nicaragua, and the functioning of such systems in this environment would be of interest.

## Immunological Studies

Two workers wrote to ask for samples of material for immunological studies. Material was supplied purified from Squalus acanthias, Cetorhinus maximus and Raja clavata.

Dr. Karl Meyer was supplied with material from Squalus, and compared it immunologically with H.F. (host factor), an uronic acid-free and sialic acid-free sulphated mucopolysaccharide prepared from the allantoic fluid of embyonated eggs, either normal or infected with influenza virus. This material was described by Norwegian workers, (Haukenes et al 1965) and was found, unlike keratan sulphate, to be antigenic in man and in rabbit. Lorenzan sulphate from Squalus acanthias was found, like keratan sulphate 11, to be immunologically inactive (Meyer et al , 1967). Meyer concluded that Lorenzan sulphate was probably more closely related chemically to KS11 (keratan sulphate, type 11) than to chick allantoic fluid saccharide (CAFS).

The other worker to ask for samples for this purpose was Dr. Michael Heidelberger, New York University Medical School. Dr. Heidelberger found that material from Cetorhinus maximus precipitated part of the antibody from Type XIV antipneumococcal serum. This implies that terminal non-reducing D-galactosyl groups are present.

## Ageing Changes in Cartilage from Squalus acanthias compared with Mammalian Cartilage

The results of the analysis for hexosamine in Squalus pectoral cartilage show that from the very young (foetal - S.acanthias is viviparous and the smallest specimens were dissected out) to the very old, the value for glucosamine as a percentage of the total amino sugar remains unchanged (Table 19 ). This means that keratan sulphate levels remain relatively constant during life in relation to chondroitin sulphate. The figures shown here for mammalian (nasal septum) cartilage show a different picture, like that described by others, e.g., Shetlar and Masters (1955) for mammalian systems. The ageing of nasal septum cartilage shows a centro-symmetric pattern when histological transverse sections are stained by metachromatic dyes. The central region appears necrotic, with a few dead or dying cells and fairly large lacunae. The intermediate zone is more active - the cells are more numerous and viable, whilst the outer layers, nearest to the perichondrium have (even in old animals) many young and vigorous cells. Thus we would expect the turnover of ground substance in the central 'aged' zone to be much less than that which borders it on either side. Ageing seems to consist of the loss of cells from this zone, and as ageing of the animal proceeds, this zone becomes progressively larger. Since it contains most of the keratan sulphate, as judged by the glucosamine (Table 20 ) and uronic acid figures, this zone cannot be expected to change its composition greatly with further ageing, but rather to extend its influence by increasing in size, over the analytical figures for the whole cartilage. At the same time, this of course affects the mechanical properties of the material which becomes less pliant in consequence of these changes. The same sort of

mechanical effect must be happening in the elasmobranch cartilage, for while the ground substance does not change ats nature chemically as far as these studies show, it does become much more calcified with age.

Simunek and Muir (1972) studying pig articular cartilage, found very little change in proteoglycan composition during adult life. Larsson and Heinegard (1975) studied ageing in bovine cartilage and found lower contents of keratan sulphate in the very young compared with the very old. They reported that the proteoglycans from foetal cartilage were of larger size than those from the older age groups. Keratan sulphate is now known in connective tissue to be part of a hybrid proteoglycan (Partridge and Elsden, 1961; Seno et al, 1965; Tsiganos and Muir, 1967). It seems possible that only one type of core protein exists, and that the ageing changes represent a shift in the biosynthetic activity of the cells in terms of their glycosylating activity which might be expected to shift further towards keratan sulphate chain production rather than chondroitin sulphate. A further possibility is that there is a shift in lysosomal degradative activity leading to the higher proportion of keratan sulphate chains. This does not seem such a likely possibility, as the two chain types do not compete for the same amino acid sites on the core, the one requiring serine whilst the other demands threonine and serine (skeletal keratan sulphate type II, as defined by Seno et al (1965) ). Skeletal keratan sulphate (as distinct from corneal keratosulphate) has O-glycosidic alkali-sensitive links to both serine and threonine. Heinegard and Axelsson (1977) found evidence that the keratan sulphate and chondroitin sulphate occupied different sites on the core protein, the keratan-sulphate enriched region being bound to hyaluronic acid

with the link proteins which, with hyaluronic acid, serve to convert this large covalent hybrid macromolecule into a massive conglomerate whose size must be enormous, and which, as a consequence, must have a dominating effect in the matter of fluid exchange in such tissues. Heinegard (1977) showed that as the size of proteoglycans decreased, so did the relative content of chondroitin sulphate, whilst the relative proportion of the hyaluronic acid-binding region and the relative content of the keratan sulphate-enriched region. He considered that proteoglycans contain a non-variable hyaluronic acid-binding region, and a keratan sulphate-enriched region and a chondroitin sulphate-enriched region of variable size. What sort of basic variations might occur in the protein core is not clear; its size does vary, but whether this is due to post-translational cleavage, or to sub-unit assembly, is not known. Since the smaller material is keratan-sulphate-enriched, and as this increases relatively with age, it is tempting to speculate that the former hypothesis is favoured.

## Sialic Acid and Cartilage

In the elasmobranch cartilage only very small amounts of sialic acid were found analytically. In mammalian cartilage however, Table 20 indicates that sialic acid occurs in substantial amounts, and appears to increase with age and with the 'ageing' layers in a manner similar to glucosamine. This reflects, probably, what is well known, namely that sialic acid is part of the keratan sulphate complex of mammalian skeletal proteoglycan. The uv absorption curves, first observed after proteolytic digestion of cartilage under differing pH conditions, are readily explained by the presence of degradation products of sialic acids under the conditions used.

Berggard and Odin (1958) studied the transformation and degradation of sialic acids in aqueous solution heated at different pH values, and found a maximum stability at pH4 to 5. At more acid or alkaline conditions, they are degraded by comparatively mild treatment. One of the alkaline degradation products is 2-carboxy-pyrrole which has a maximum absorbance at 262 n m in neutral solution, and varying somewhat with pH. Chromogens also appear to result from N-acetylhexosamine released during the alkaline treatment of sialic acids. These ultraviolet absorbing peaks are only manifested during proteolytic digestion when the high background absorbance due to protein is removed. When the process was repeated using elasmobranch cartilage, these peaks were not observed, presumably because the levels of sialic acid concentration were not high enough.

The ageing changes in elasmobranch cartilage described here are the subject of a paper (included at the end) on the subject.

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Table of Figures.

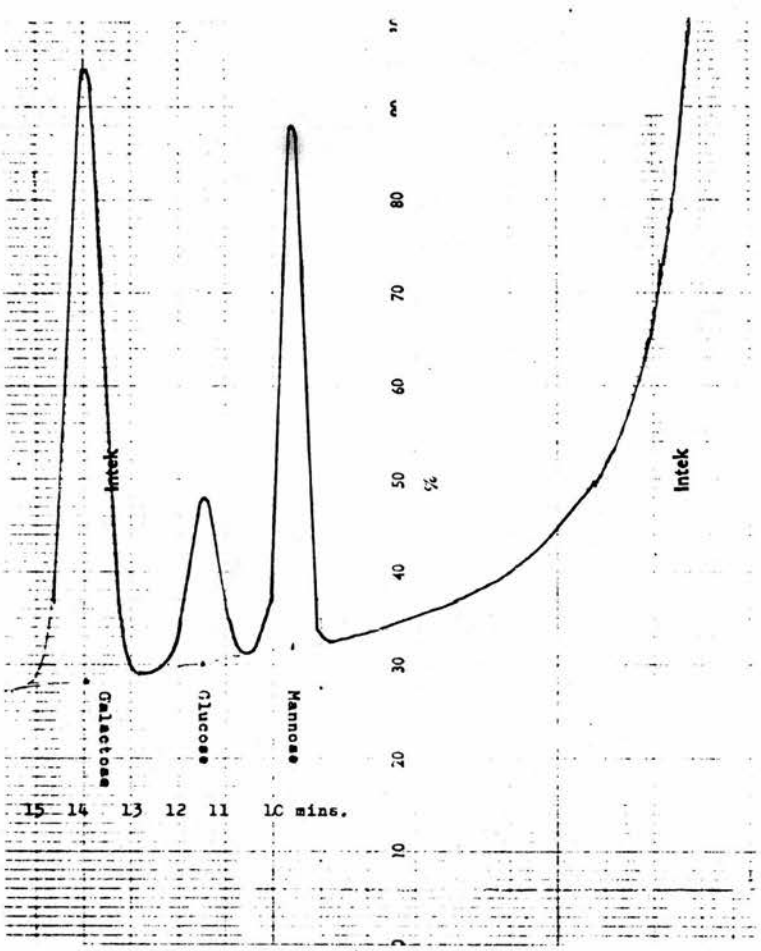



Fig.1

Figure 1. Separation of neutral sugars as butyl boronate derivatives on G.L.C.

Butyl boronate derivatives of the alditol acetates ( 5ug total sugars in 5ul) were applied to the column in pyridine. The column was 2 m x 0.5cm ov 17 on Gas chrom Q, used on a Pye 104 gas chromatograph. The oven temperature was 200°C and the detector temperature, 240°C. The carrier gas was nitrogen, and the flow rate was 35 ml/min. The area under the peak was measured by the product of the height X the width at half the height.

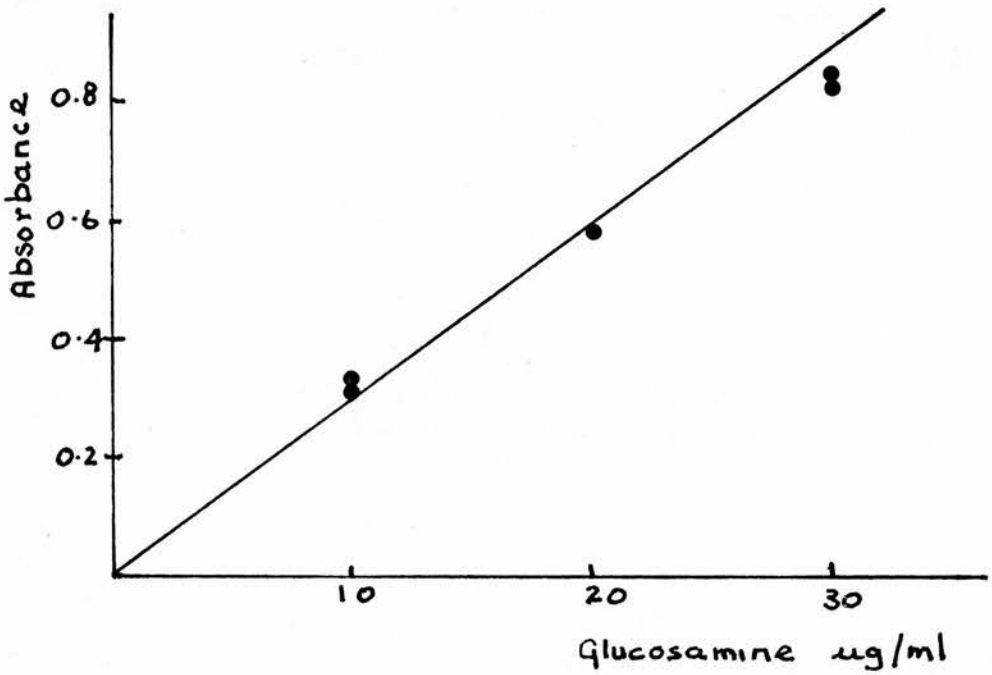



Fig.2

Figure 2. Amino sugar standard curve using the method of Gatt & Berman (1966).

Glucosamine hydrochloride was used as the standard, and one ml aliquots of aqueous solutions containing 10, 20 or 30 ug of the free base were used. The absorbancies were measured at 530 nm in 1 cm cuvettes.

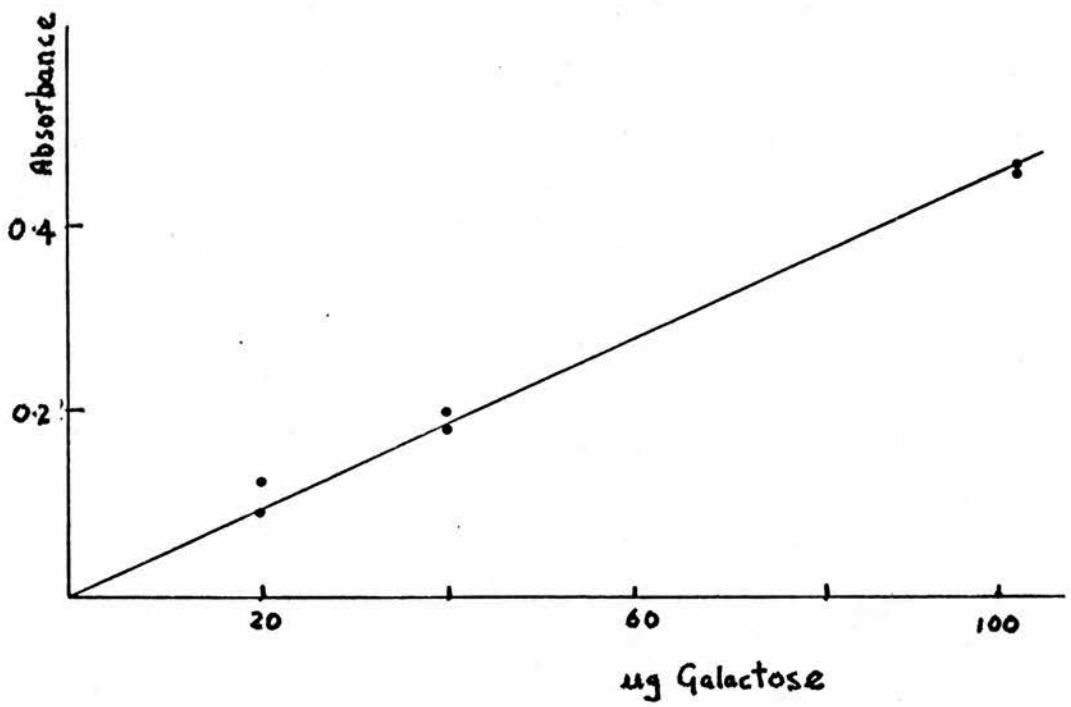



Fig.3

Figure 3. Neutral sugar calibration curve using the anthrone method of Trevelyan & Harrison (1952) as modified by Dische (1955).

D-Galactose was used as the standard sugar, and 0.5 ml aliquots of solutions containing 20, 40 or 100 ug of sugar were layered on to the pre-cooled anthrone reagent prior to mixing. Absorbancies were read at 640 nm in 1 cm cuvettes.

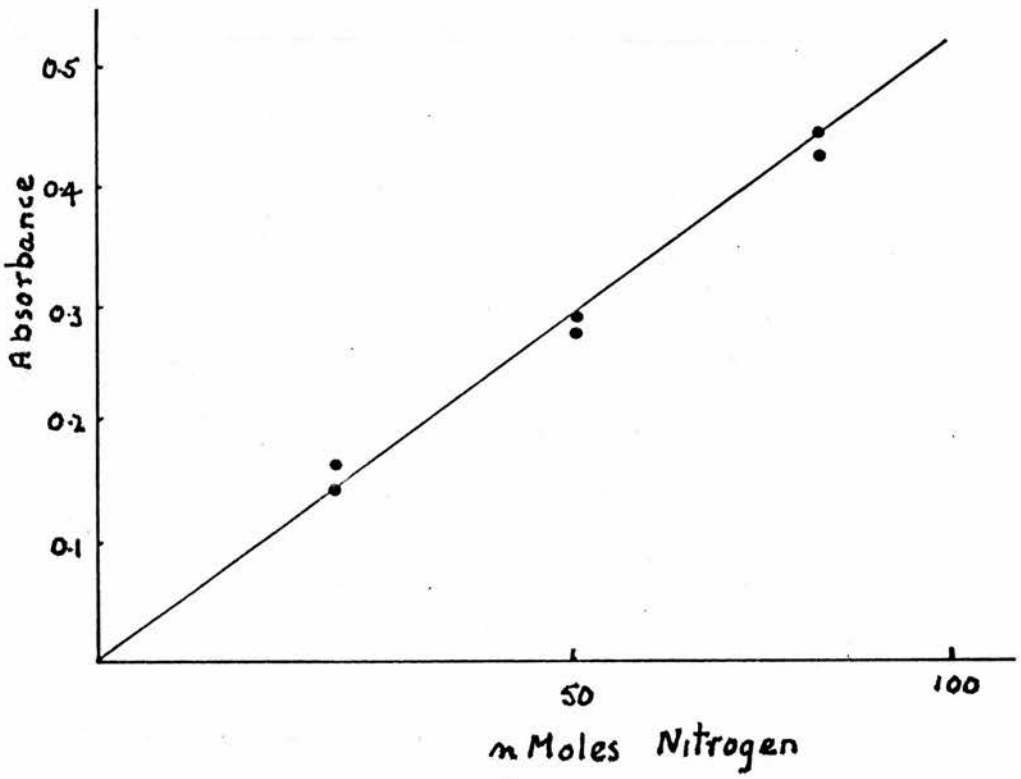



Fig.4

Figure 4. Calibration curve of nitrogen estimation by the method of Jaenicke (1974).

The standard used was a solution of Analar ammonium sulphate at a concentration containing 200 nmole nitrogen/ ml. After adding the chromophore forming reagents and mixing (see page 27), the absorbancies were measured at 578 nm. Pre-oxidation of the protein samples was with perchloric acid at 215°C in a heated aluminium block.

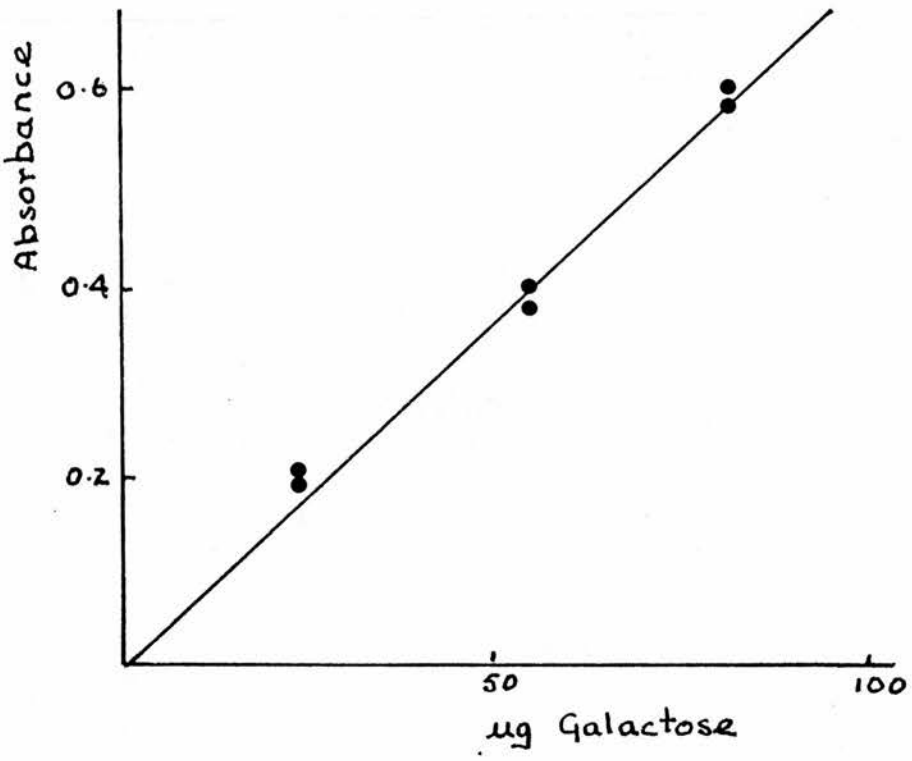



Fig.5

Figure 5. Calibration curve of neutral sugar estimation by the method of Dubois et al., (1956).

Aliquots (0.5 ml) of standard galactose solutions containing up to 100 ug galactose were pipetted into test tubes followed by the reagents. Absorbancies were read in 1 cm cuvettes at 490 nm.

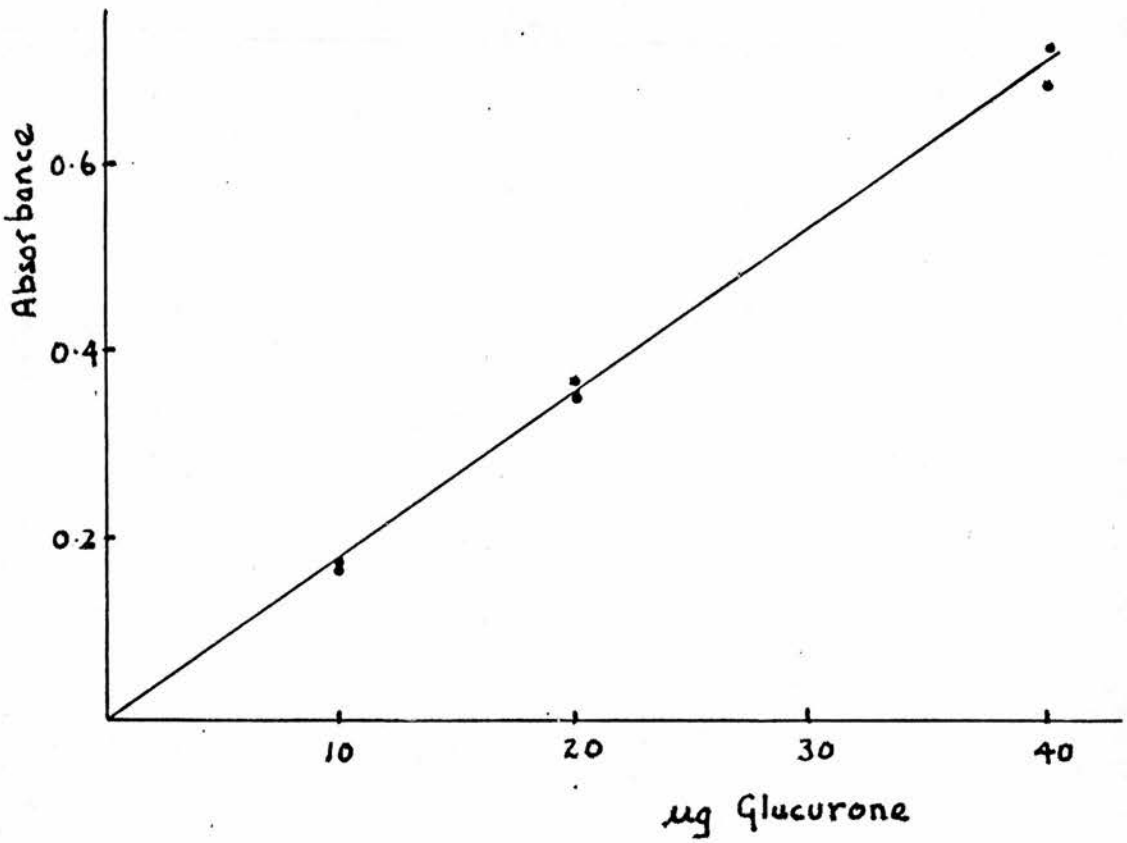



Fig.6

Figure 6. Calibration graph of uronic acid assay by the method of Bitter & Muir (1962).

The reference standard was D-glucurono-lactone. The standard solutions containing up to 40 ug glucuronolactone ( glucurone) in one ml of water were layered on top of the pre-cooled sulphuric acid reagent, then gently shaken prior to incubation. After cooling and adding the carbazole reagent, the absorbancies were read in 1 cm cuvettes at 530 nm.

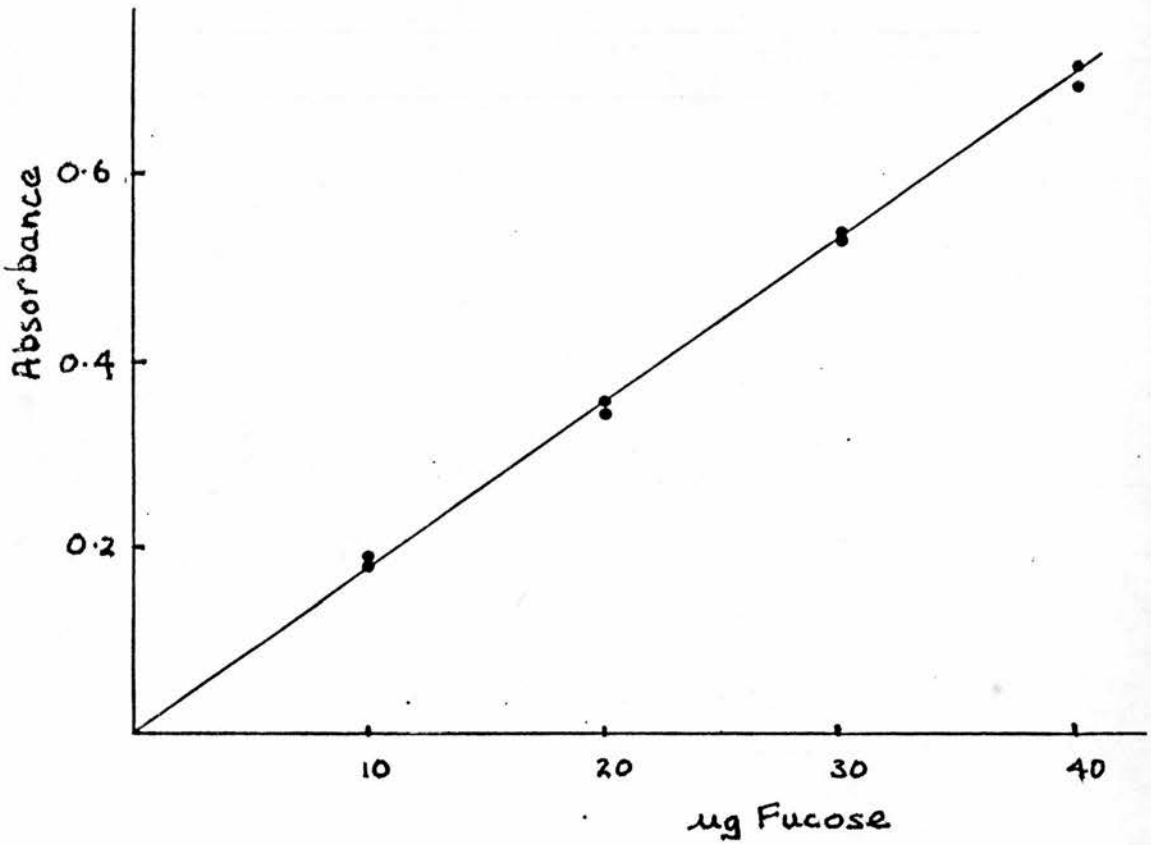



Fig.7

Figure 7. Calibration curve for the assay of fucose by the method of Gibbons (1955).

Solutions (1.0 ml) containing 10 - 40 ug of the methyl pentose were pipetted into a test tube followed by the reagents. After the incubations, the absorbancies were read at 410 nm in 1 cm cuvettes.

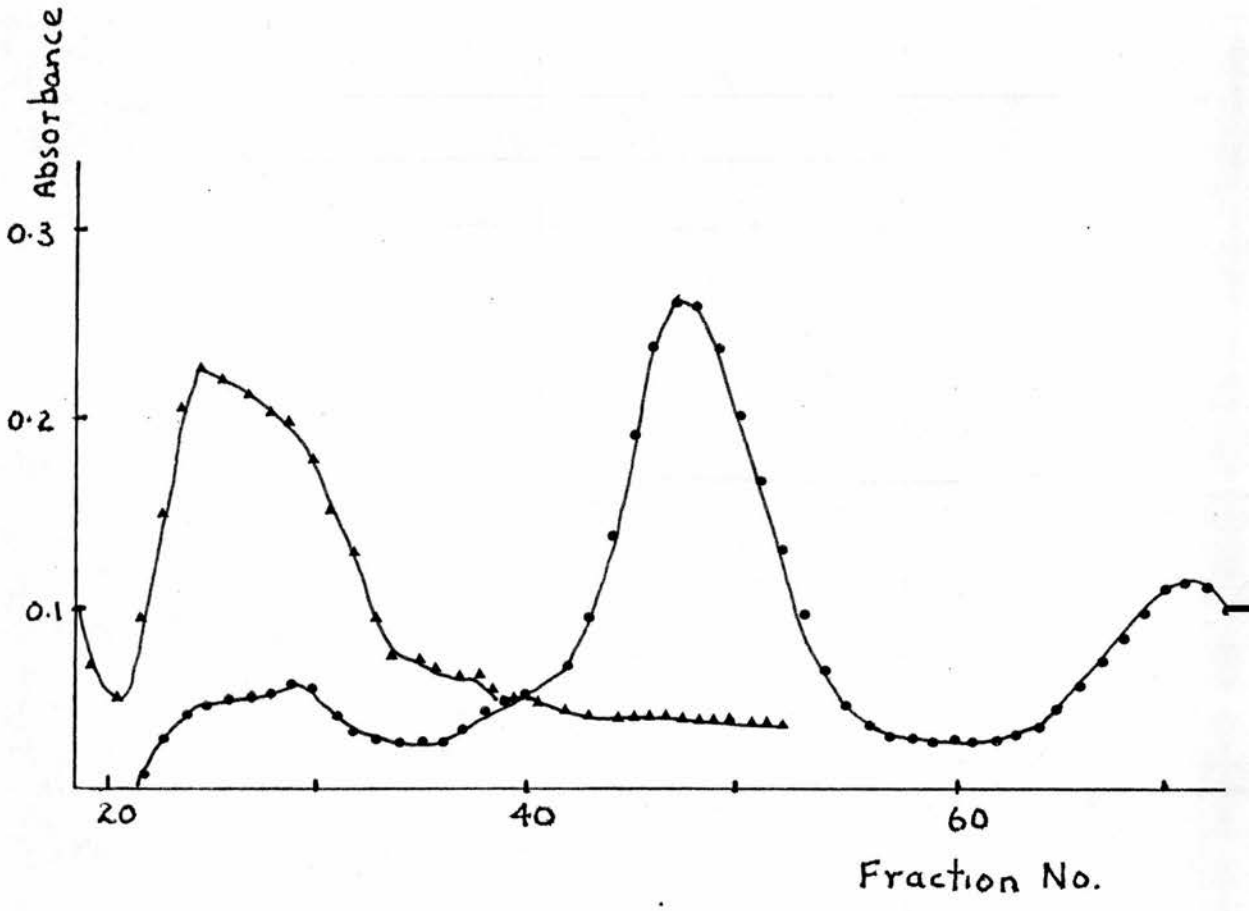



Fig.8

Figure 8. Purification of papain digests of Squalus acanthias Lorenzini jelly by gel filtration on Sephadex G 100.

Samples (150 mg) of the dialysed freeze-dried papain digests of the native Lorenzini jelly from Squalus acanthias were applied in 10 ml of 1% NaCl to a column of Sephadex G 100, 42 x 5 cm, packed with 1% NaCl. The column was eluted with 1% NaCl, and 10 ml fractions were collected. Aliquots were analysed for protein by measuring the absorbance at 280 nm in a 1 cm silica cuvette, and for neutral carbohydrate by the phenol/$H_2SO_4$ method using 0.1 ml aliquots of the fractions and measuring the absorbance after the colorimetric reaction at 490 nm.

Protein ● ● ●

Carbohydrate ▲ ▲ ▲

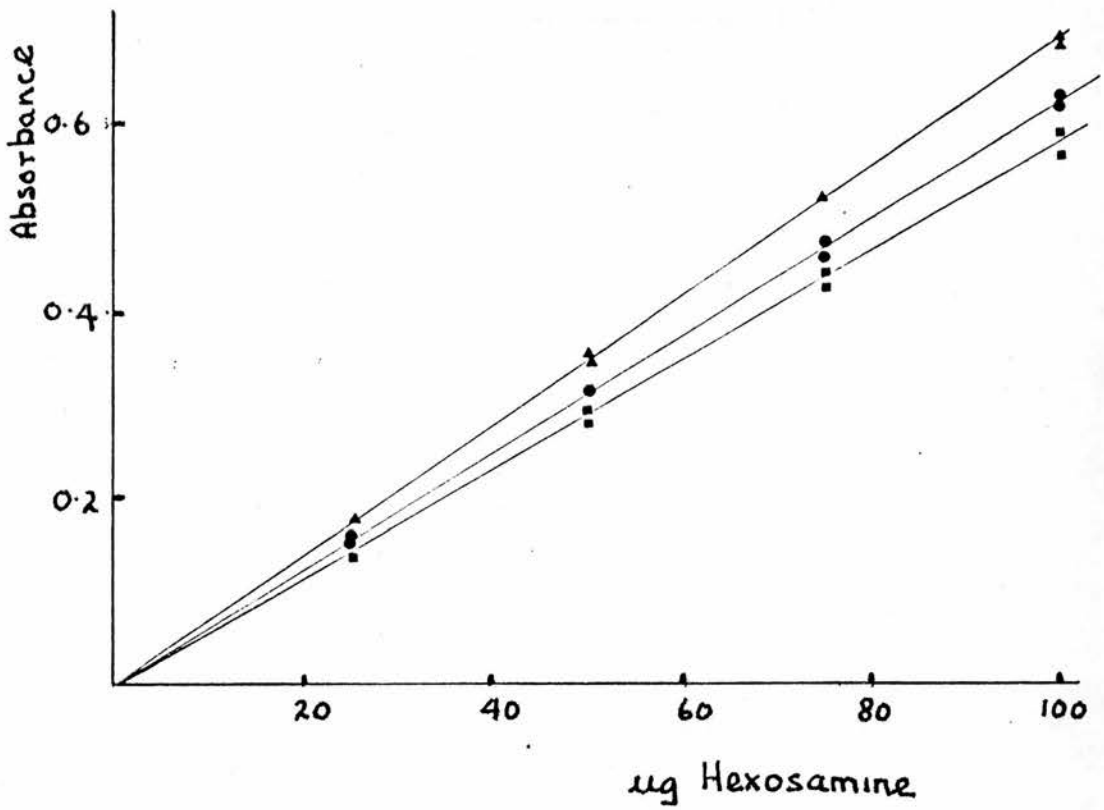



Fig.9

Figure 9. Calibration graphs of amino sugar colorimetric analysis by the method of Gardell (1953).

The standards used were solutions of glucosamine hydrochloride containing 25, 50, 75 and 100 ug of the free base per ml. The tubes were sealed in different ways to find the optimum colour development from the heating period with the acetylacetone reagent. The concentration of sodium carbonate was changed from 1.25 M as in the published method, to 1.5 M resulting in increased sensitivity. Absorbancies were finally read at 530 nm in 1 cm cuvettes.

Tubes covered with Parafilm and fan cooled ▲ ▲ ▲

Tubes covered with marbles ● ● ●

Tubes open ■ ■ ■

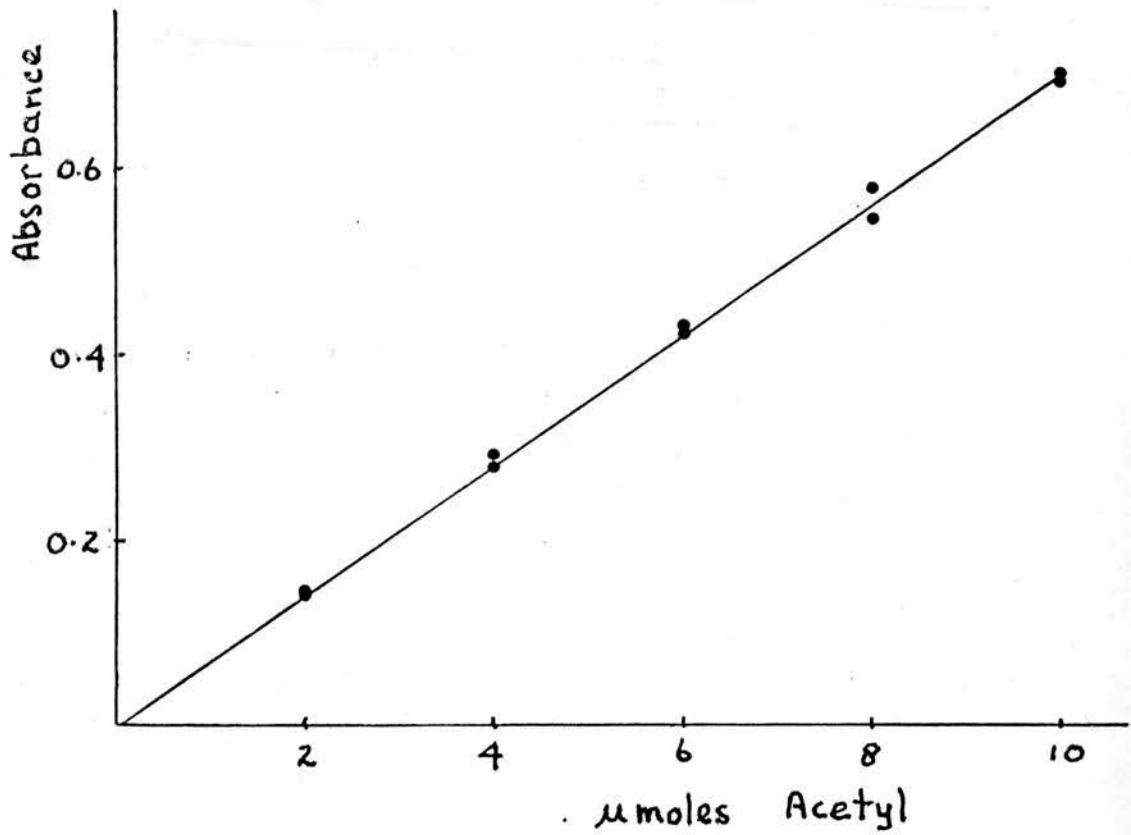



Fig.10

Figure 10. Calibration curve of acetyl group determination by the method of Ludowieg and Dorfman (1960).

The standards were prepared using methanol redistilled from molecular sieve. Ethylacetate ( 5mM ) in methanol:water ( 50:50 v/v) was the standard. All measurements were made against a 2M HCl in methanol blank using B.O.C. gaseous HCl dried by an $H_2SO_4$ wash. Absorbancies were read at 520 nm in a 1 cm cuvette.

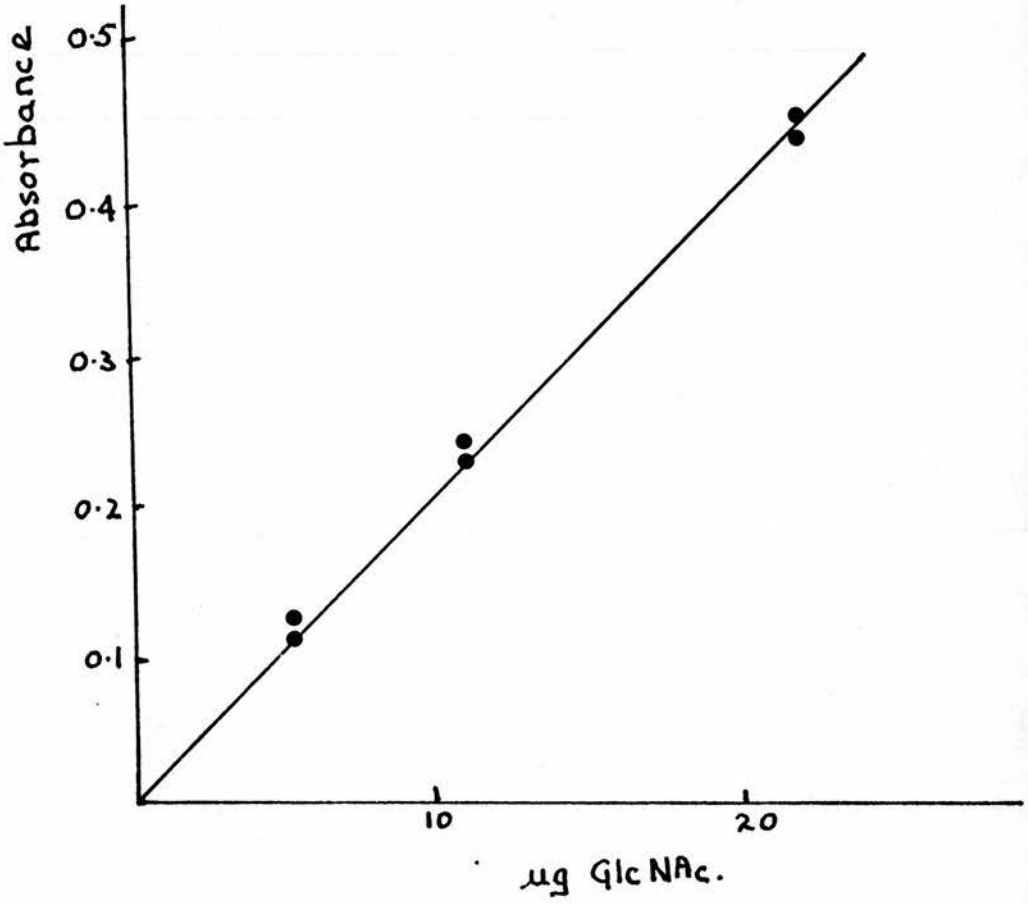



Fig.11

Figure 11. Calibration curve for the assay of N-acetyl glucosamine by the method of Reissig (1955).

Standard solutions of synthetic N-acetyl-glucosamine ( 50 ug/ml ) were used and amounts up to 25 ug were pipetted out into test tubes for the calibration. The volume of all the standard solutions was made up to 1.0 ml with water before adding the colorimetric reagents. Absorbancies were read at 585 nm in 1 cm cuvettes.

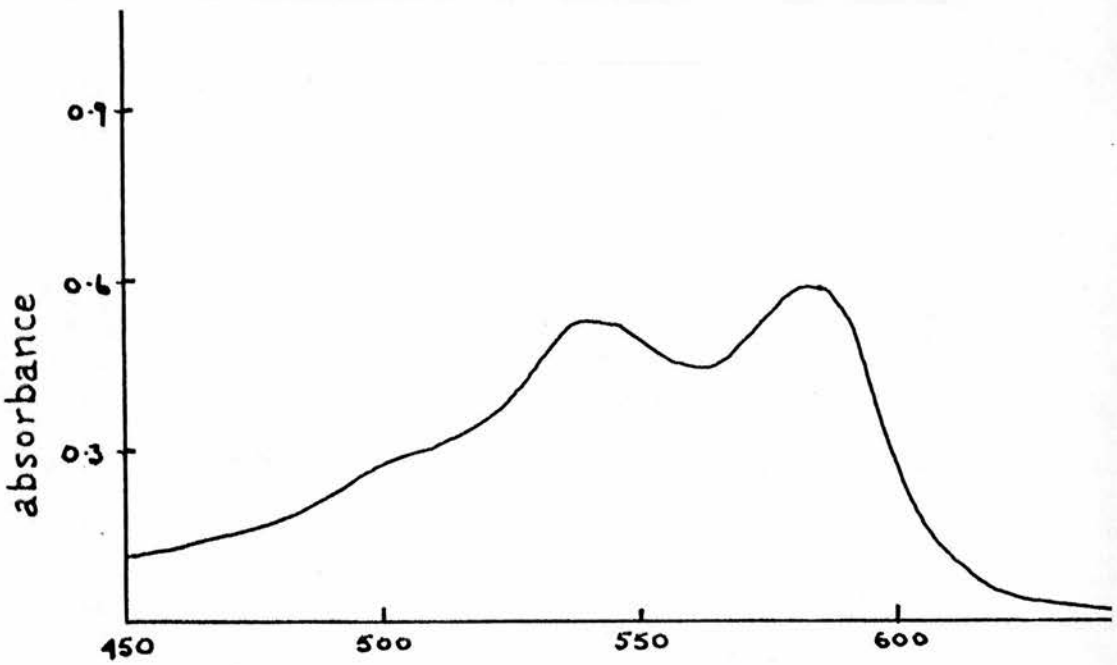



Fig.12

Figure 12. Spectrum of the Morgan-Elson chromophore.

The spectrum was plotted in a Unicam SP 800 recording spectrophotometer after reacting 30 ug N-acetyl-glucosamine according to the method of Reissig (1955). One cm cells were used, and the blank was 1 ml water treated in the same way as the standard N-acetyl-glucosamine.

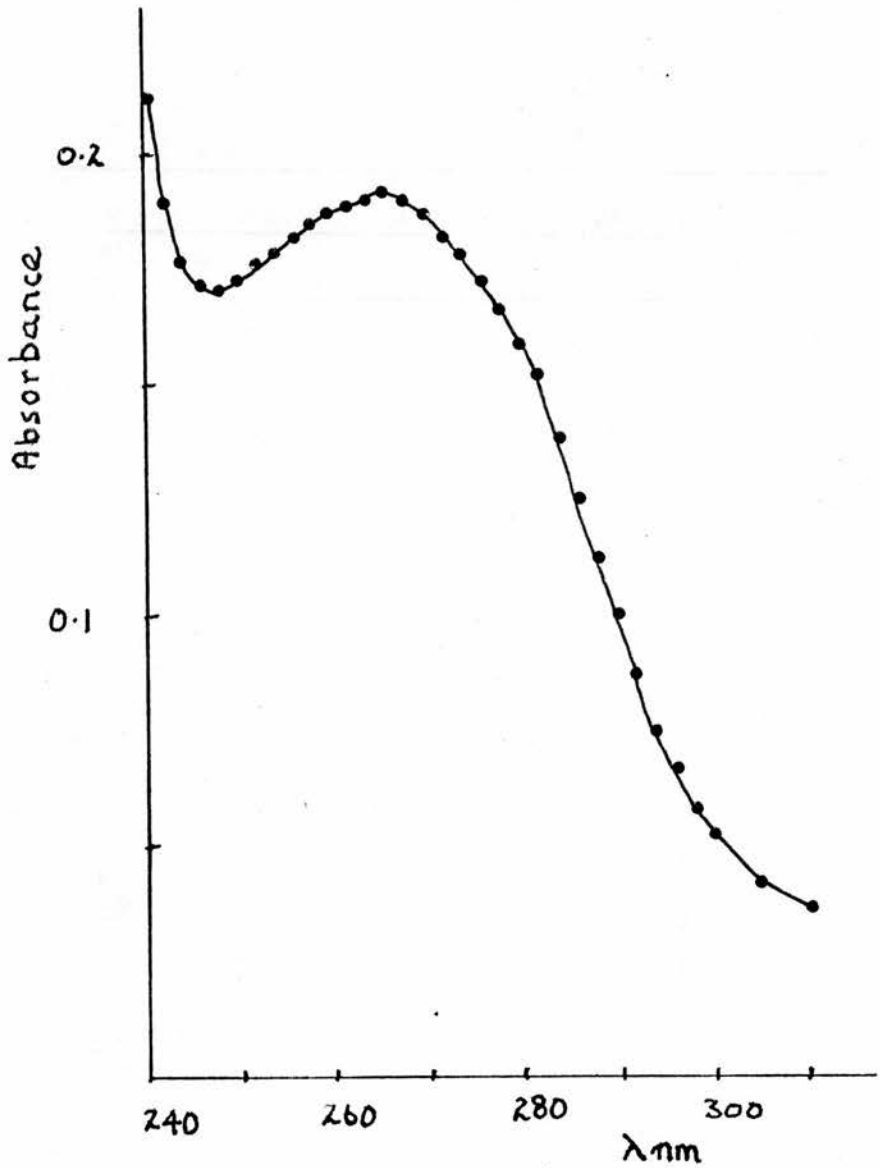



Fig.13

Figure 13. Ultra-violet absorption spectrum of the native Lorenzini jelly of Squalus acanthias.

One gram of native jelly was shaken with water and made up to 10 ml, and read in 1 cm silica cells in a Unicam SP 500 spectrophotometer against water as a blank.

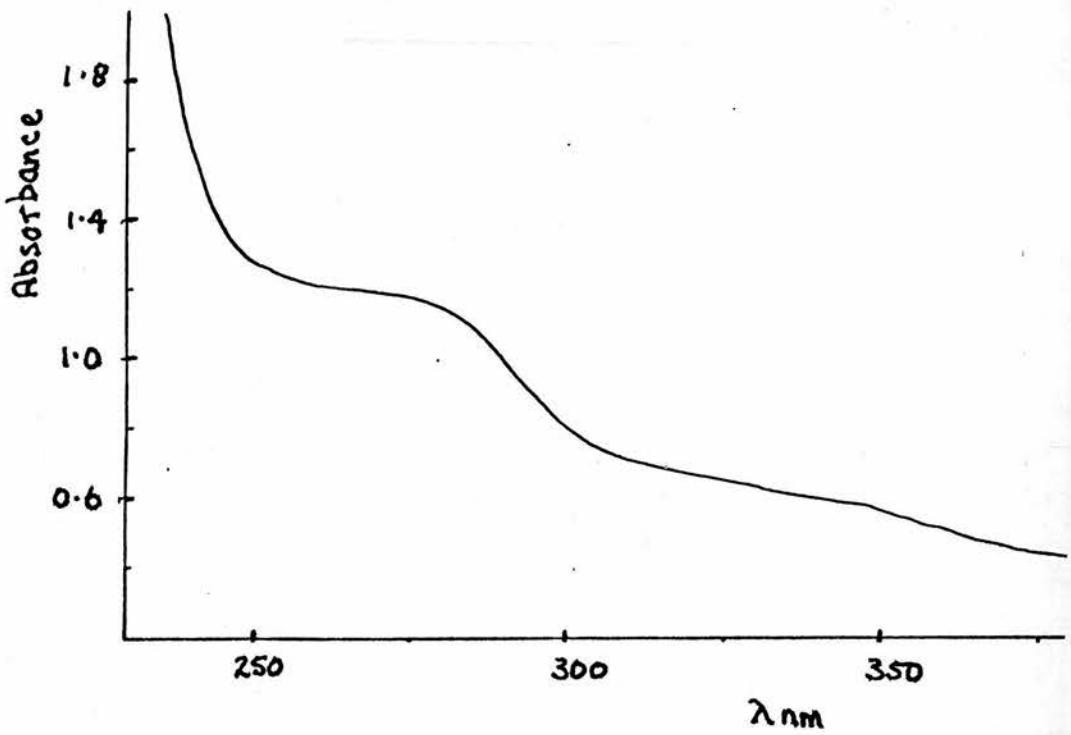



Fig14

Figure 14. Ultra-violet absorption spectrum of the native Lorenzini jelly of Galeorhinus galeus.

One gram of native jelly was shaken with water and made up to 10 ml, and read in 1 cm silica cells in a Unicam SP 800 recording spectrophotometer against water as a blank.

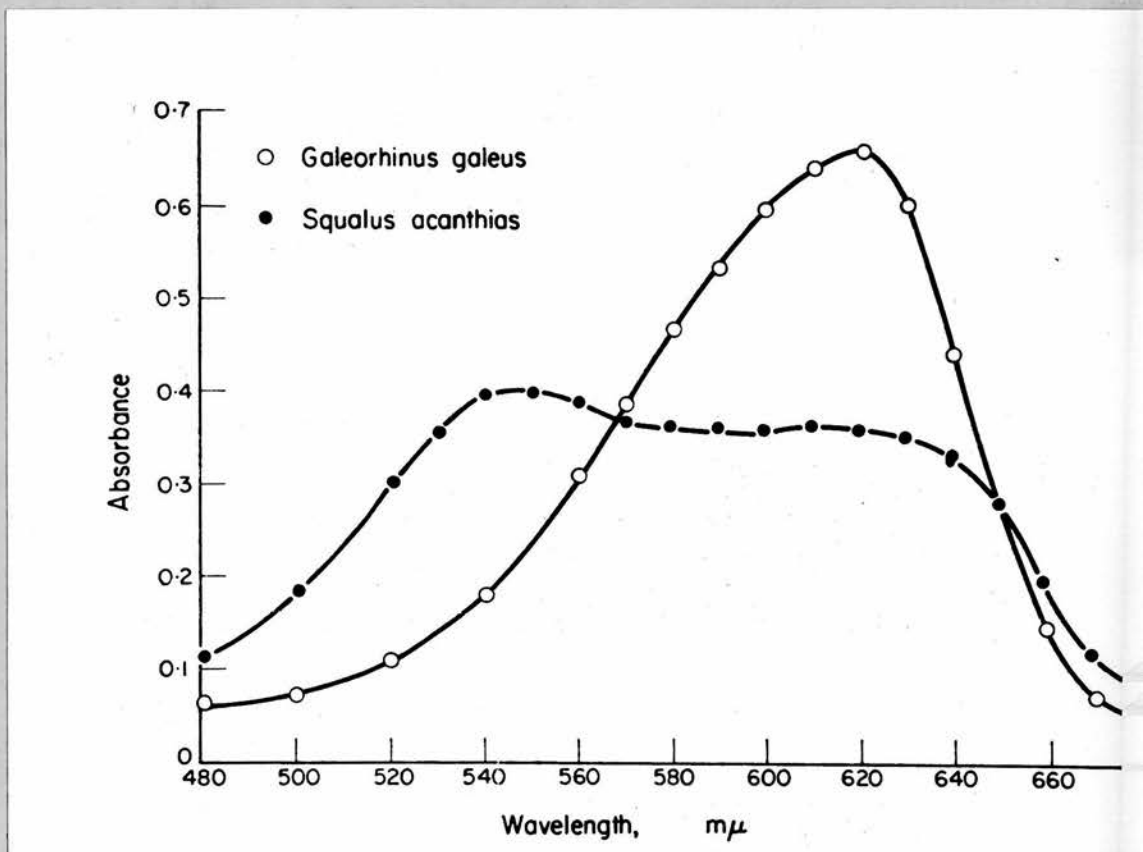

0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Absorbance
Galeorhinus galeus
Squalus acanthias
480
500
520
540
560
580
600
620
640
660
Wavelength, mμ

Fig. 15

Figure 15. A comparison of the metachromatic activity of Lorenzini jelly from Squalus acanthias with that from Galeorhinus galeus.

Solutions of the native jelly ( 1.5 ml of a 10% aqueous solution) were mixed with equal volumes of 1% w/v Azur A in M/100 HCl, and the spectra measured in 1 cm cells against water as a blank.

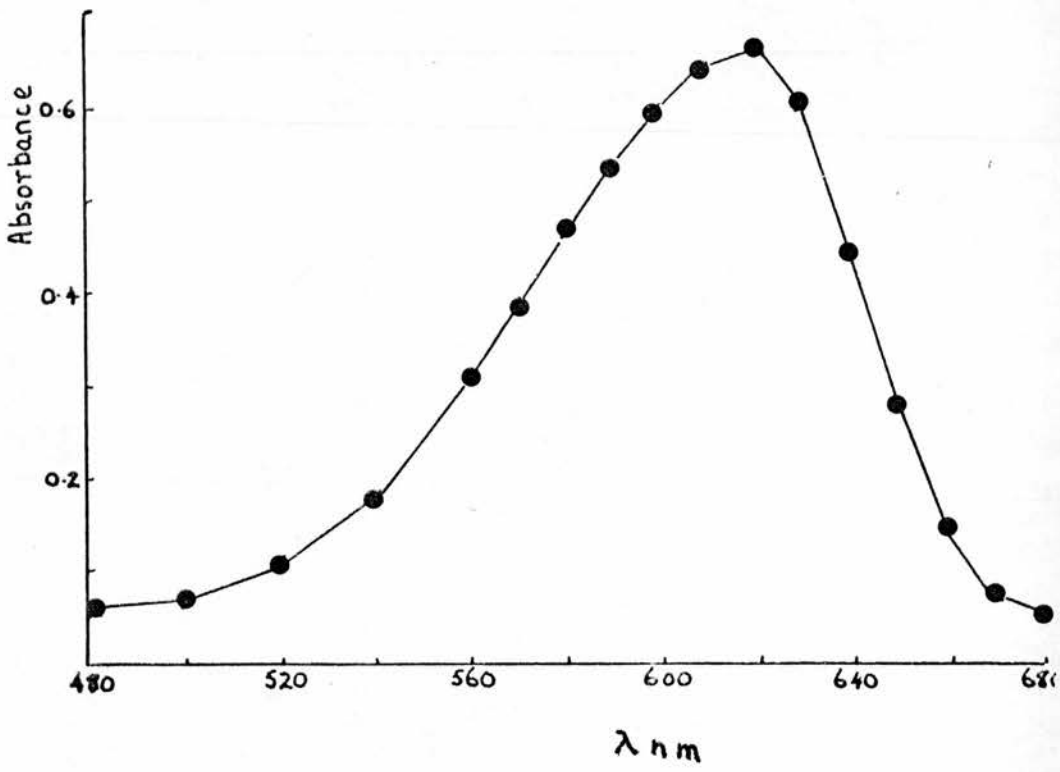



Fig.16

Figure 16. The absorption spectrum of Azur A.

A 1% solution of Azur A, 1.5 ml dissolved in M/100 HCl was mixed with an equal volume of water and read in an SP 500 spectrophotometer in 1 cm cells using water as a blank.

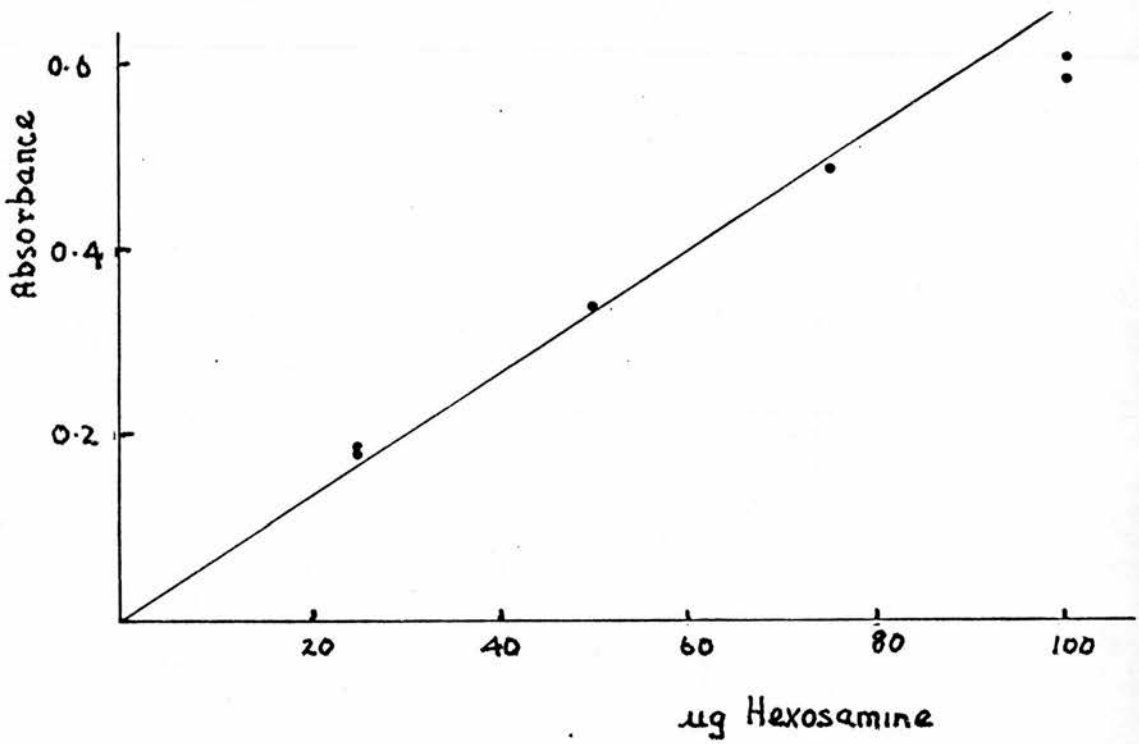



Fig 17

Figure 17. Calibration graph of hexosamine analysis by the method of Gardell (1953).

A standard solution of glucosamine hydrochloride was used, and aliquots pipetted to give from 25 to 100 ug of free base/ ml in duplicate for calibration of all the Gardell column assays. Absorbancies were measured at 530 nm in 1 cm cells.

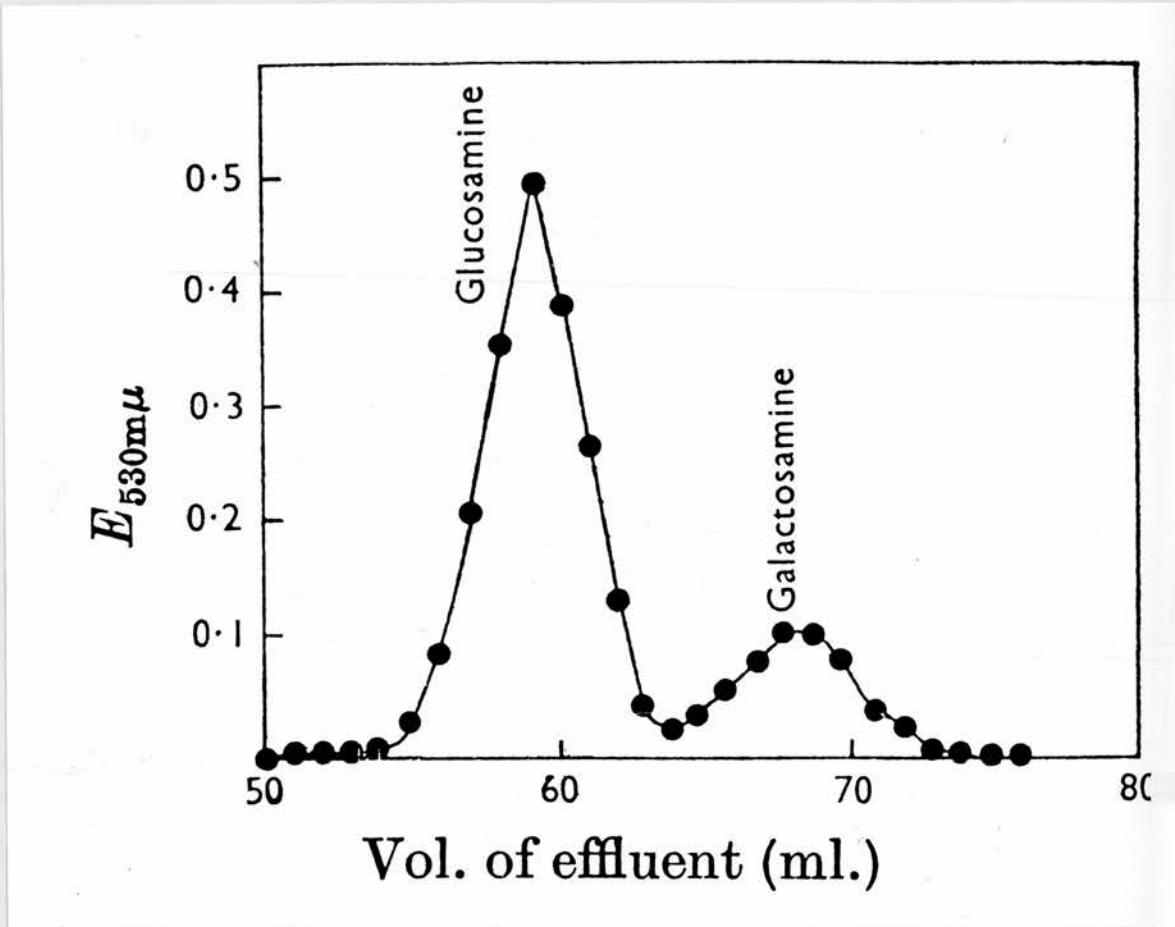

Glucosamine
Galactosamine
$E_{530m\mu}$
0·5
0·4
0·3
0·2
0·1
50
60
70
80
Vol. of effluent (ml.)

Fig. 18

Figure 18. Separation and analysis of the individual hexosamines on Dowex 50 ( 200 - 400 mesh) ion exchange resin, ( Gardell, 1953).

Columns of 40 x 0.6 cm were packed in 0.3 M HCl, and the amino sugar hydrolysate, containing a total of 150 ug of hexosamine was applied in 0.5 ml of 0.3 M HCl, and eluted using the same solvent. One ml fractions were collected and analysed for amino sugars. Glucosamine HCl was used as a standard, and a correction was applied for the lower colour yield given by galactosamine.

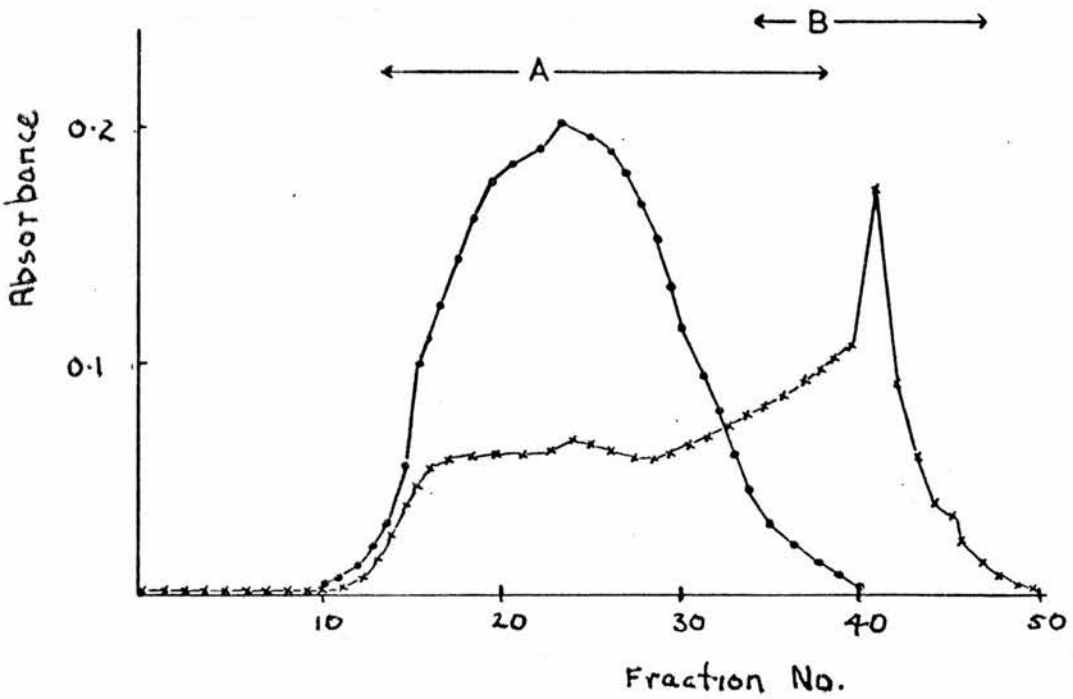



Fig.19

Figure 19. Fractionation of Squalus acanthias papain digest on columns of Sephadex G50.

Columns, 30 x 2.5 cm, were packed in water and 5 ml aliquots of the crude digest containing 35 mg of the original dried jelly, were applied and eluted with water. Fractions of 4 ml were collected and 0.25 ml aliquots analysed for neutral carbohydrate by the anthrone method ( Dische, 1955), and for protein by the method of Lowry (1951).

The region marked 'A' was metachromatic, whilst that marked 'B' gave a positive chloride test.

Carbohydrate peak • • •

Protein peak + + +

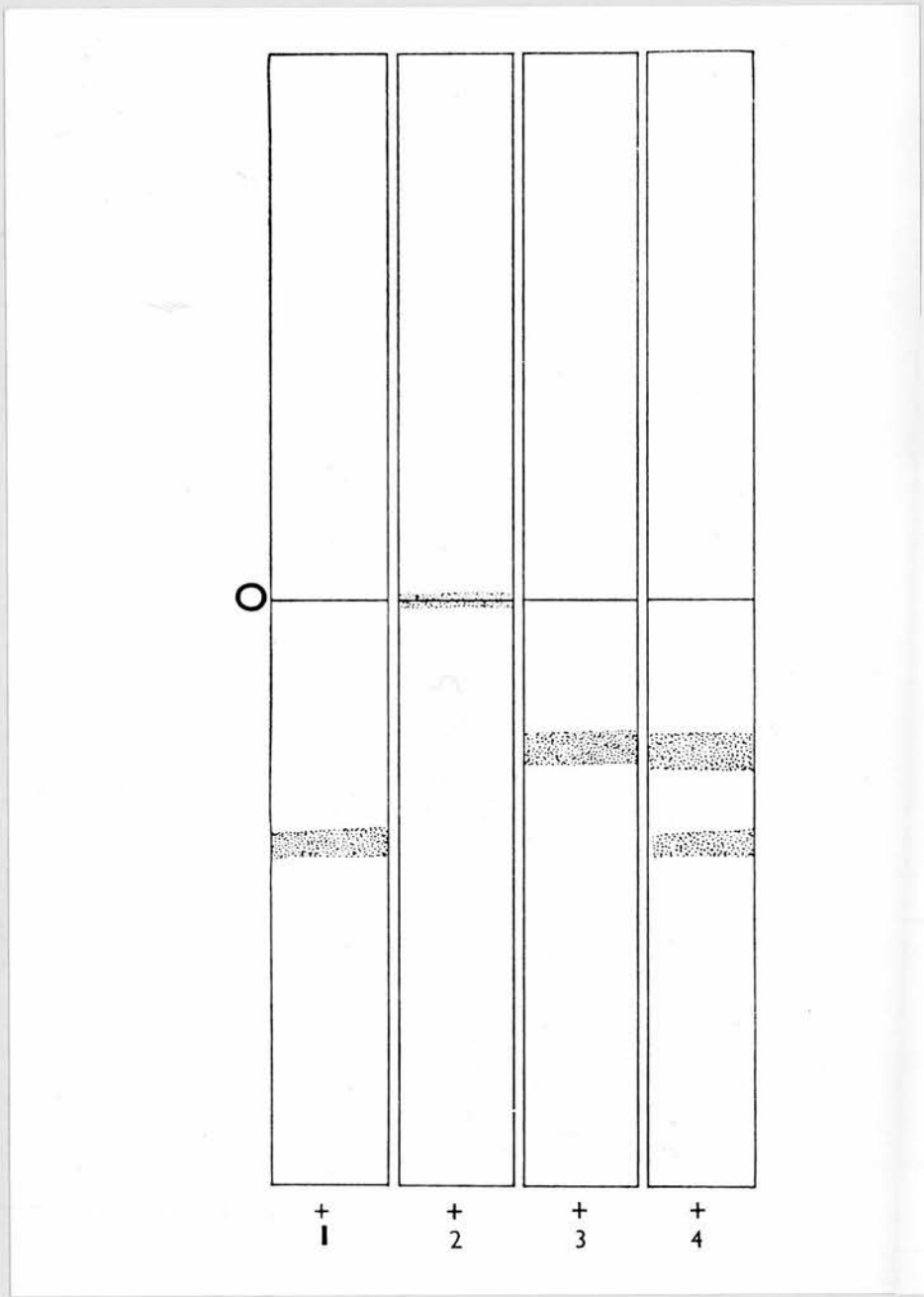



Fig. 20

Figure 20. Paper electrophoresis of the Sephadex G-50 purified Lorenzini glycopeptide material.

Electrophoresis was carried out in 0.1 M veronal buffer, pH 8.6 for 90 minutes at 30 volts/cm. The strips were dried and stained in 1% Azur A in 0.01 M HCl. Excess dye was removed by washing with 1% acetic acid.

0 - Origin

1 - Chondroitin sulphate

2 - Native Lorenzini jelly

3 - Purified Lorenzan sulphate

4 - A mixture of 1 + 3

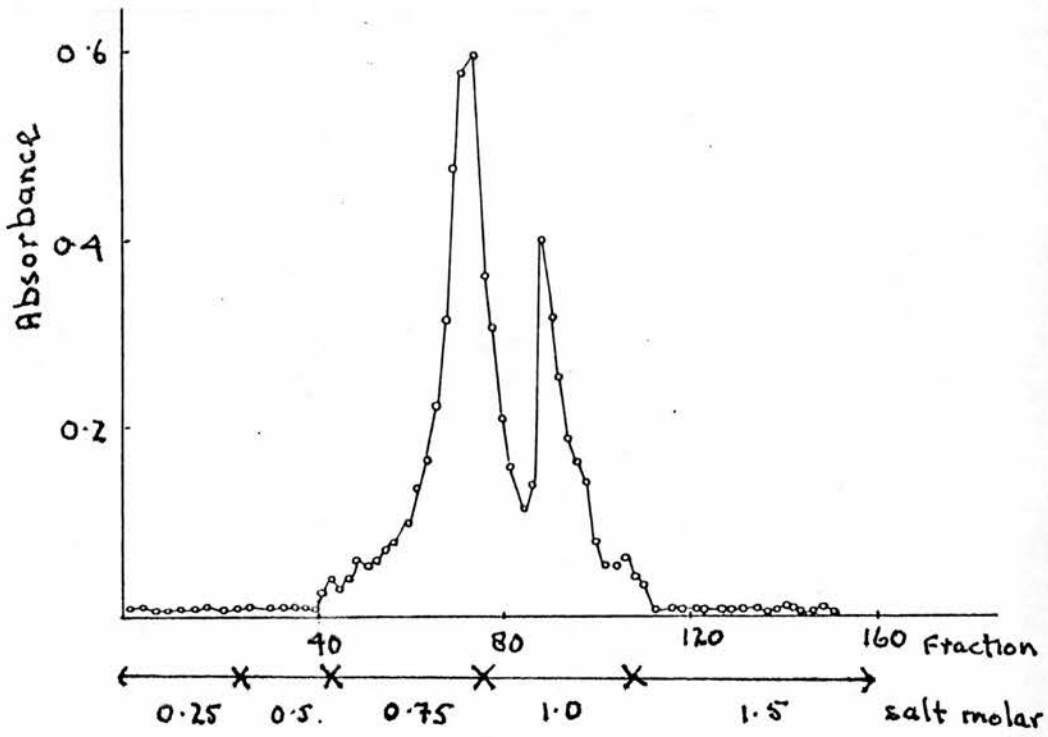

Absorbance
0·6
0·4
0·2
40
80
120
160
Fraction
0·25
0·5
0·75
1·0
1·5
salt molar

Fig.21

Figure 21. Fractionation of Squalus acanthias glycopeptide on DEAE Sephadex A 50 columns.

Columns of DEAE Sephadex A 50, 40 x 2.5 cm, were packed in 0.1 M Tris buffer, pH 8.0. A sample, 100mg, of the Sephadex G 100 purified glycopeptide was applied in 10 ml of buffer, and the column eluted with 600ml buffer containing five steps of increasing salt concentration as shown. Fractions of 4 ml were collected, and aliquots analysed for neutral carbohydrate by the anthrone method of Dische (1955).

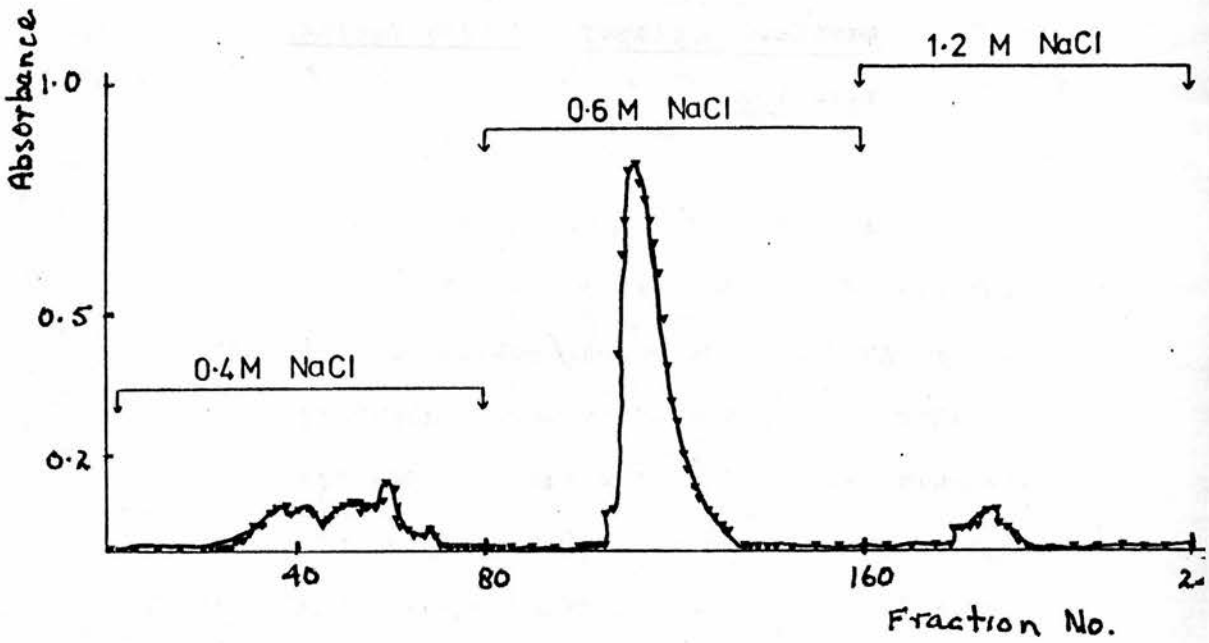

Absorbance
1·0
0·5
0·2
0·4M NaCl
0·6 M NaCl
1·2 M NaCl
40
80
160
Fraction No.

Fig.22

**Figure 22. Fractionation of Sephadex G 100 purified glycopeptide from Squalus acanthias.**

A preparative large scale column of DEAE Sephadex 70 x 6 cm was packed in 0.1 M Tris/HCl buffer, pH 8.0. The sample applied was 850 mg of Sephadex G 100 purified Lorenzini glycopeptide dissolved in 10 ml of the buffer. The column was eluted with a total of 9 litres of the pH 8.0 buffer in three separate salt concentration steps as indicated. Fractions of 25 ml were collected and aliquoys analysed by the phenol/$H_2SO_4$ method of Dubois (1956) for carbohydrate. The three carbohydrate peaks obtained were pooled, dialysed and freeze dried.

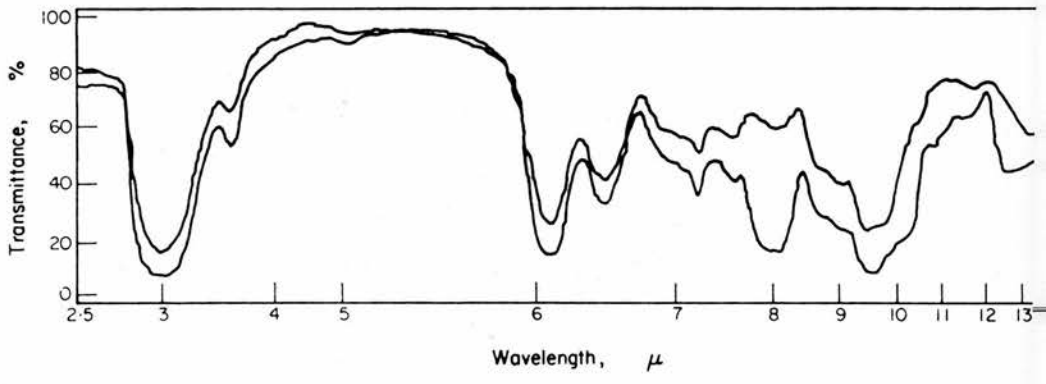

Transmittance, %
100
80
60
40
20
0
2·5
3
4
5
6
7
8
9
10
11
12
13
Wavelength, μ

Fig. 23

Figure 23. Infra-red spectrum of Lorenzan sulphates.

The Sephadex G 100 ( or G 150 in the case of Galeorhinus galeus) purified glycopeptide, 2 mg, was dried over NaOH in vacuo and mixed with 200 mg dry KBr, and a pellet made under pressure with a vacuum pump connected to the system. The infra-red spectrum of the transparent disc was measured in a Perkin-Elmer Model 257 spectrophotometer plotting % Transmittance against wavelength .

Upper spectrum Galeorhinus galeus

Lower spectrum Squalus acanthias

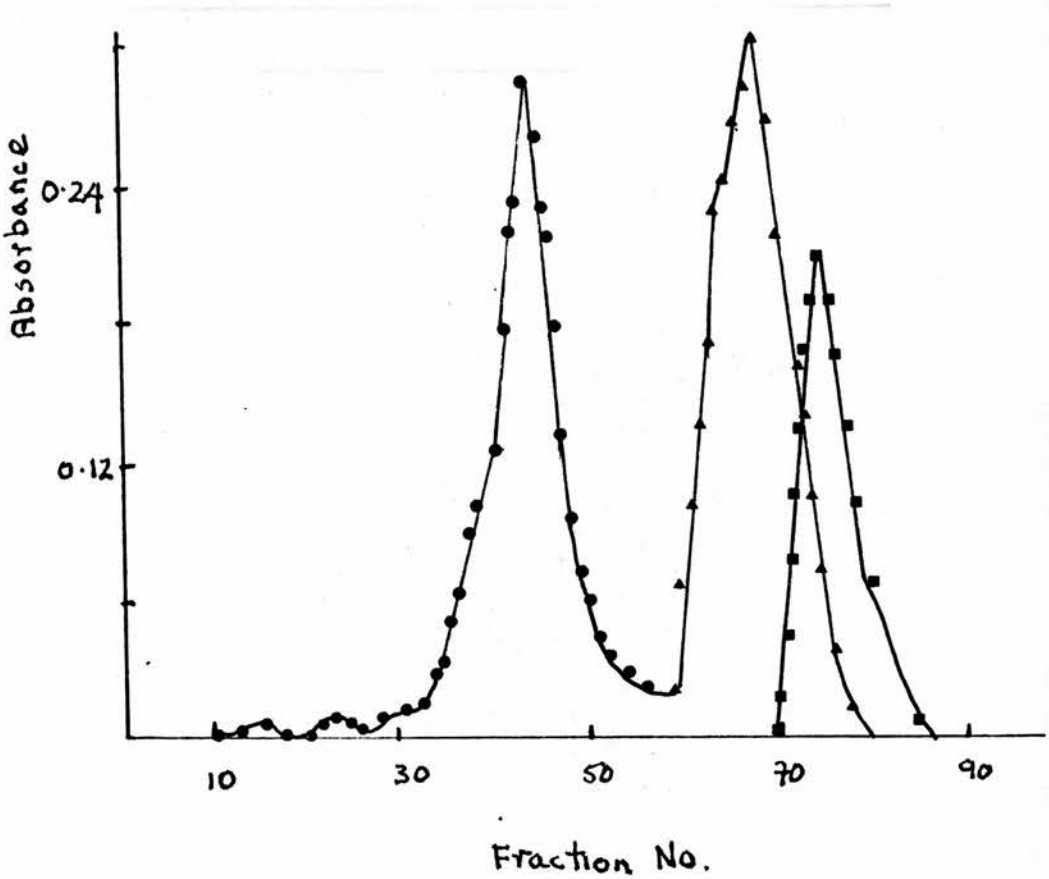



Fig.24

Figure 24. Calibration of Ultrogel AcA34 gel filtration column.

A column of Ultrogel AcA34 mixed agarose/ polyacrylamide gel, 80x2.6 cm was packed in 0.1 M Tris/HCl buffer, pH 7.5, 0.2 M in NaCl and 1mM with respect to EDTA. Samples of known proteins (10 mg of each) were applied in buffer, and run separately by ascending chromatography to locate their positions on the column. Aliquots of the 5.0 ml fractions collected on elution by the buffer were analysed for protein by the method of Bradford (1976)

Peak 1 Thyroglobulin M.W. 670,000 ● ● ●

Peak 2 Bovine Serum Albumin M.W. 67,000 ▲ ▲ ▲

Peak 3 Myoglobin M.W. 17,000 ■ ■ ■

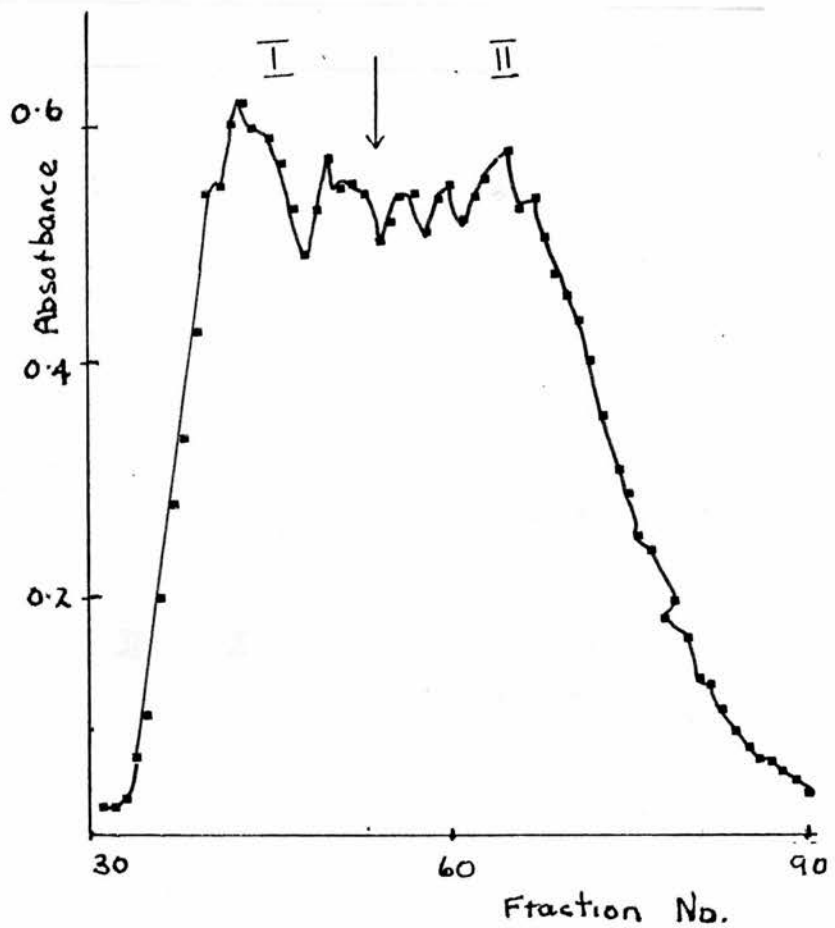



Fig. 25

Figure 25 Fractionation of L.S./ S.a./ G100 /DE-2 on Ultrogel AcA34 columns.

The column, 80 x 2.6 cm was used with the pH 7.5 buffer that the column was packed in, as in Fig. 24. The sample applied in 5.0 ml of the buffer was 120 mg of the glycopeptide. Analysis of aliquots of the 5.0 ml fractions was by the method of Dubois (1956) for neutral carbohydrate. The eluate was arbitrarily split into two fractions, A 34-1 and A34-2 , shown as I and II in the figure. Recovery was 100.2 mg total.

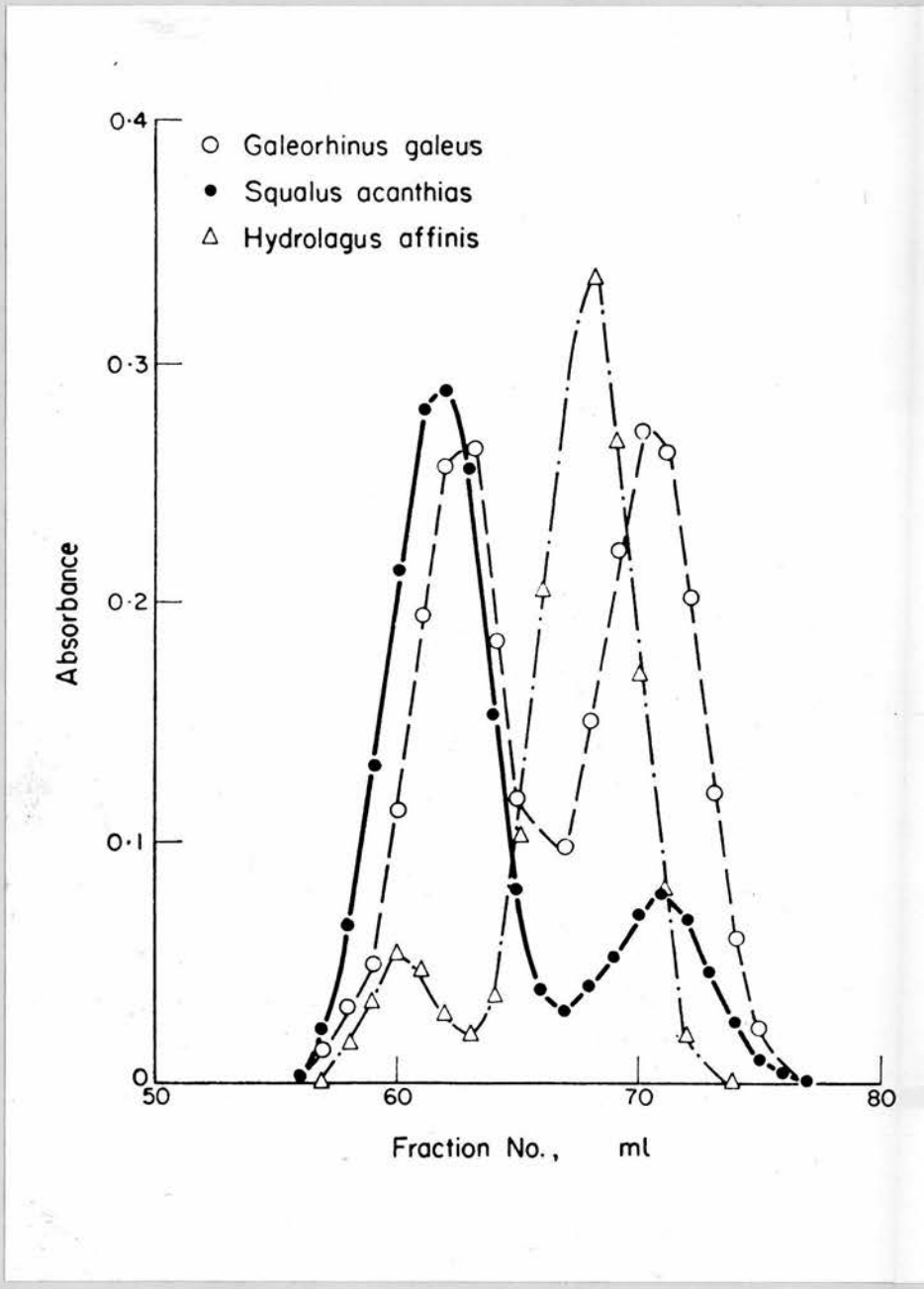

0·4
0·3
0·2
0·1
0
Absorbance
○ Galeorhinus galeus
● Squalus acanthias
△ Hydrolagus affinis
50
60
70
80
Fraction No., ml

Fig. 26

Figure 26 Hexosamine analysis on Lorenzini jelly from three species of elasmobranch.

Analysis was performed by the method of Gardell(1953). The figure shows the results of analysis on material from three species in which the wide range of ratios found can be appreciated.

Galeorhinus galeus ○ ○ ○

Squalus acanthias ● ● ●

Hydrolagus affinis △ △ △

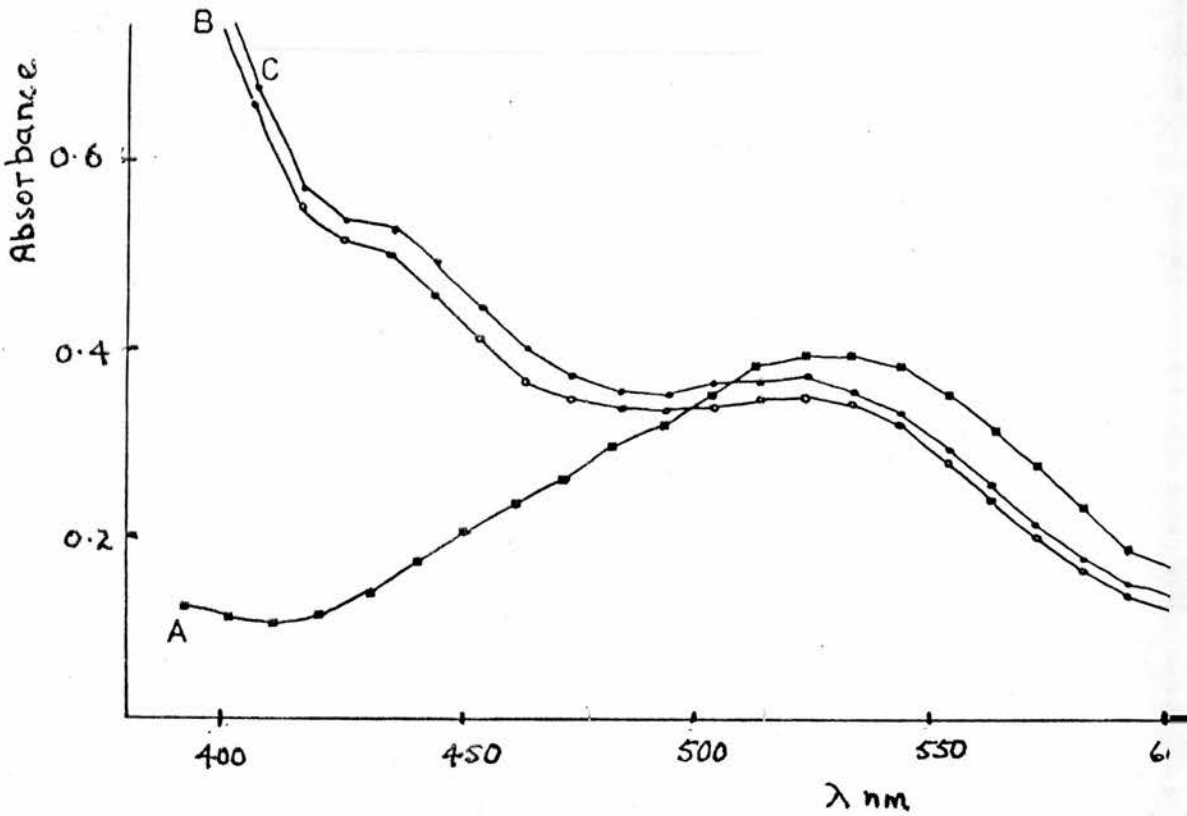



Fig.27

Figure 27 Spectra of chromophores obtained in the assay for uronic acid by the method of Bitter & Muir (1962).

The interference of galactose in the assay for uronic acid is shown in this figure. Absorbancies were measured in 1 cm cuvettes.

| | |
|---|---|
| Curve A | D- glucurone 20 ug/ ml |
| Curve B | D- galactose 200 ug/ml |
| Curve C | Lorenzini glycopeptide with equivalent galactose concentration to that in Curve B. |

Figures 28a, 28b , and 28c. Biogel P2 fractionation of the glycopeptides.

The column used was 70 x 2 cm, packed in 1% NaCl. The fraction volume was 1.5 ml, and aliquots of the fractions were analysed for carbohydrate by the method of Dubois (1956).

Fig. 28a Calibration of the column using Dextran blue (peak 1), sucrose (peak 2 ) and glucose (peak 3) All three were run separately.

Fig. 28b Fractionation of Squalua acanthias glycopeptide ( upper graph) and alkali-treated glycopeptide(lower graph). Samples (30 mg) were applied in 2.0 ml of 1% saline.

Fig 28c Fractionation of Galeorhinus galeus glycopeptide (upper graph) and alkali treated glycopeptide(lower graph). Samples (30mg) were applied in 2.0 ml of 1% saline.

The qualitative presence of Morgan-Elson chromogen was detected in the fractions of alkali degraded material only, and its position is indicated on graphs 28b and 28c (lower).

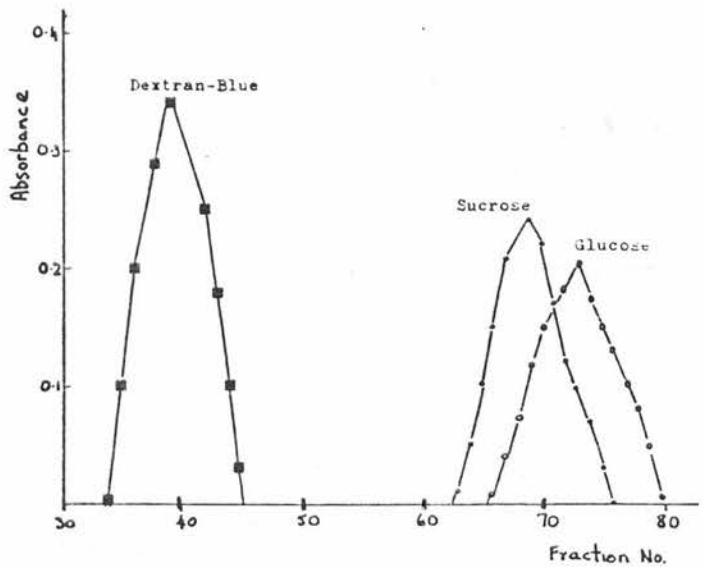

Absorbance
0·4
0·3
0·2
0·1
Dextran-Blue
Sucrose
Glucose
30
40
50
60
70
80
Fraction No.

Fig.28a

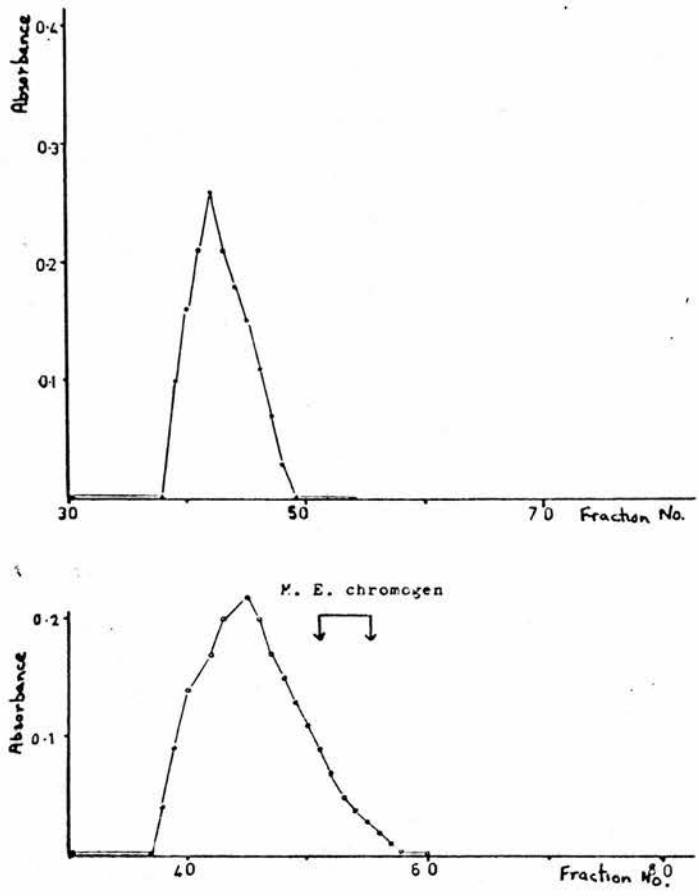

Absorbance
0·4
0·3
0·2
0·1
30
50
70
Fraction No.
M. E. chromogen
Absorbance
0·2
0·1
40
60
80
Fraction No.

Fig.28b

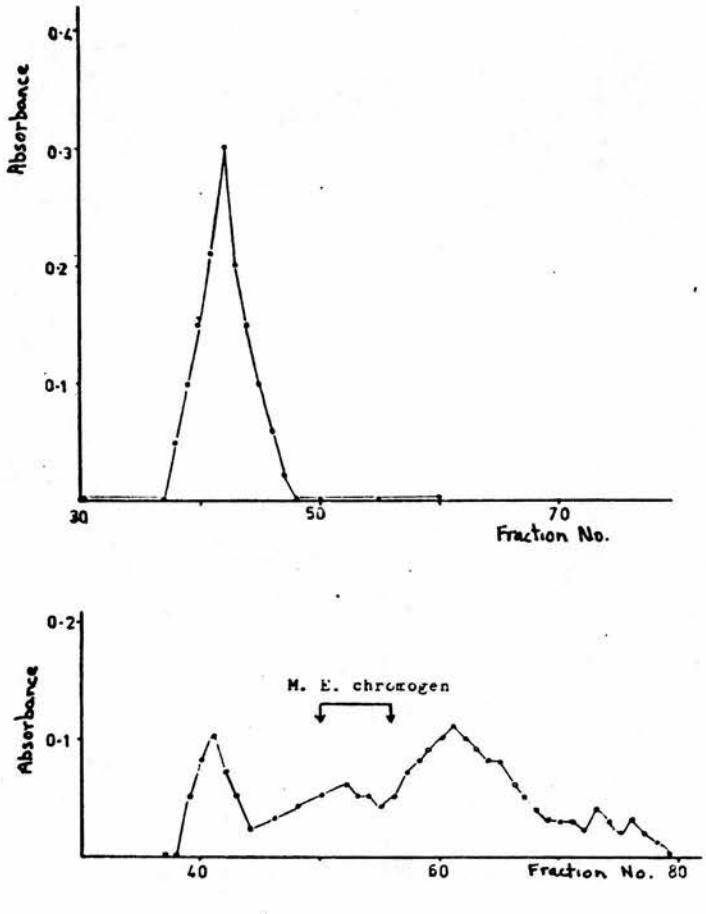

Absorbance
0·4
0·3
0·2
0·1
30
50
70
Fraction No.
Absorbance
0·2
0·1
M. E. chromogen
40
60
Fraction No. 80

Fig.28c

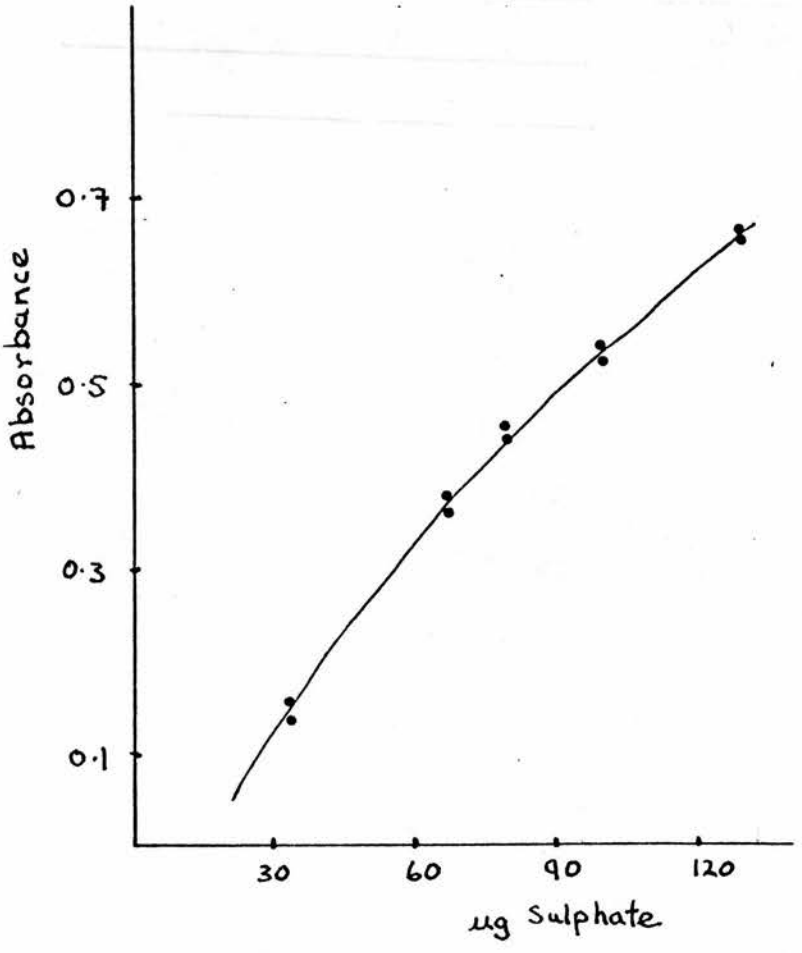



Fig.29

Figure 29 Calibration curve of sulphate determination using the method of Jones & Letham (1956)

Absorbancies were measured in 1 cm silica cuvettes. The slope is positive as the individual results of different sulphate concentrations were subtracted from the blank.

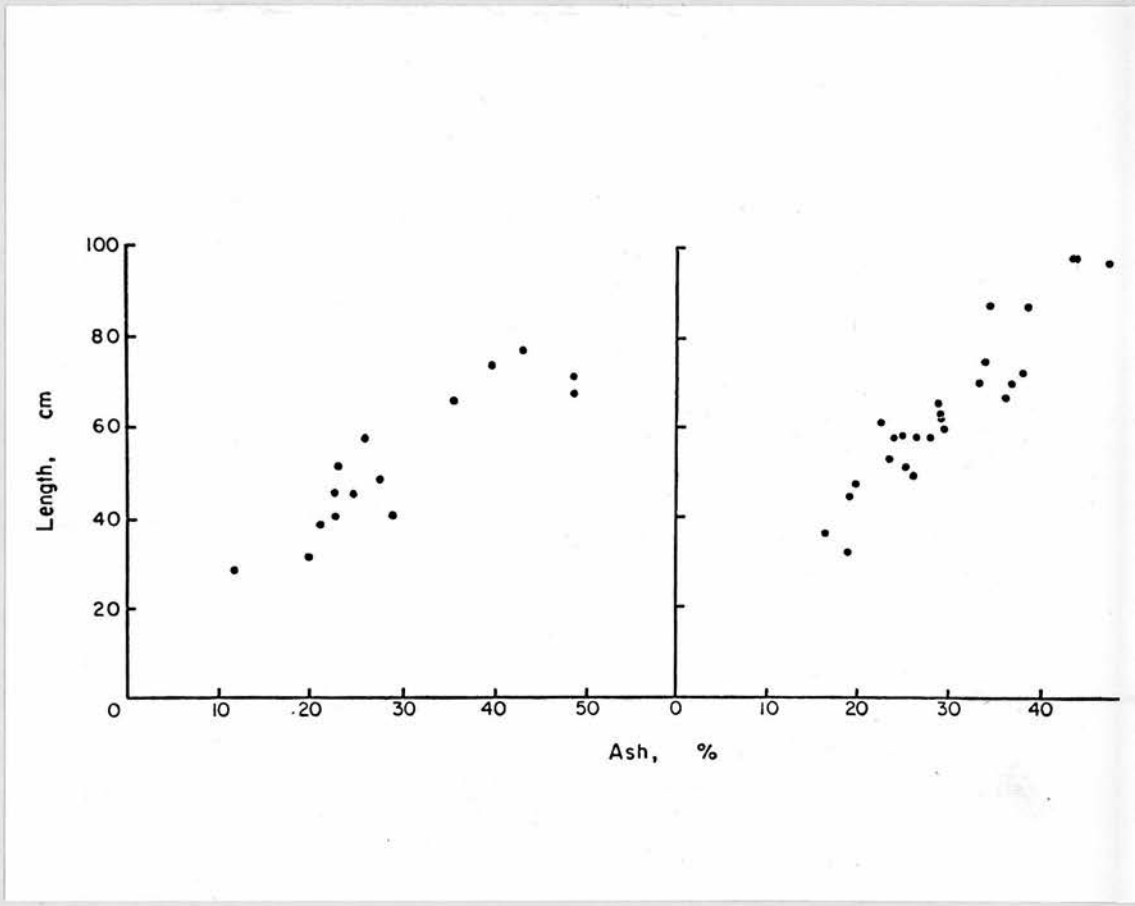



Fig. 30

Figure 30 Variations in the ash content of pectoral cartilage from Squalus acanthias.

Dried powdered cartilage ( 50 mg) was ashed at 500°C in an electric furnace to constant weight. A few drops of M/1 $H_2SO_4$ were added to convert any carbonates to sulphates prior to the heating. The figure shows the ash content plotted as a function of length of the animal.

Left, males ; Right, females.

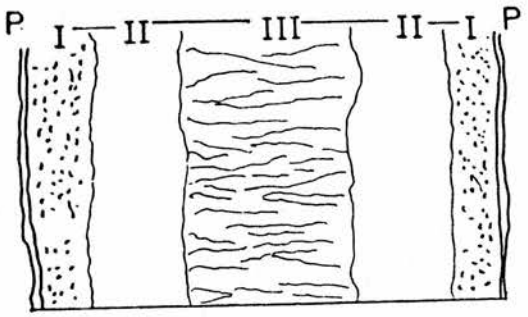



Fig.31

Figure 31 Diagram of a T.S. of horse nasal septum cartilage.

The figure shows a simplified diagram sketched from a transverse section of horse nasal septum stained at pH 2.0 with Azur A. The zones of different staining found are as described in the text.

P = perichondrium

I = outer layers

II= inner layers

III= the central cell free zone found only in older animals.

Figures 32a, 32b, 32c, 32d, and 32e.

The demonstration of the uv chromophores derived from sialic acid in cartilage.

The figure shows the uv spectra of solutions of cartilage prepared and treated in various ways derived from mammalian nasal septum (horse) compared with elasmobranch cartilage.

Fig. 32a Horse nasal septum cartilage

A.- after autoclaving and filtering.

B.- after collagenase digestion

Fig 32b The same as above treated by pepsin-trypsin digestion after autoclaving.

Fig 32c The same treated by collagenase digestion and heating.

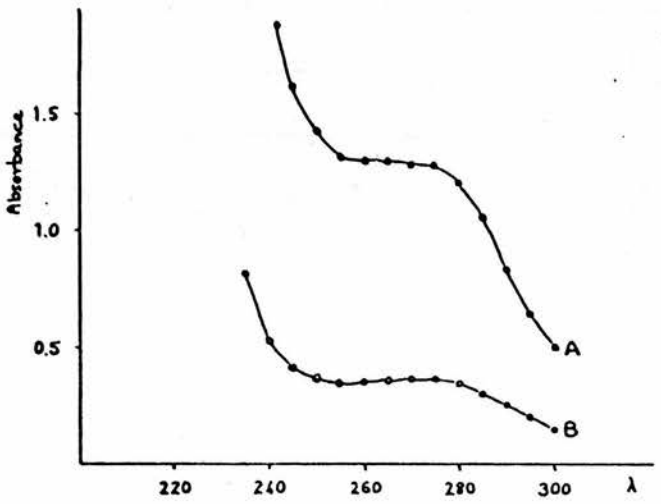
Fig 32d The same as above A. After collagenase digestion.

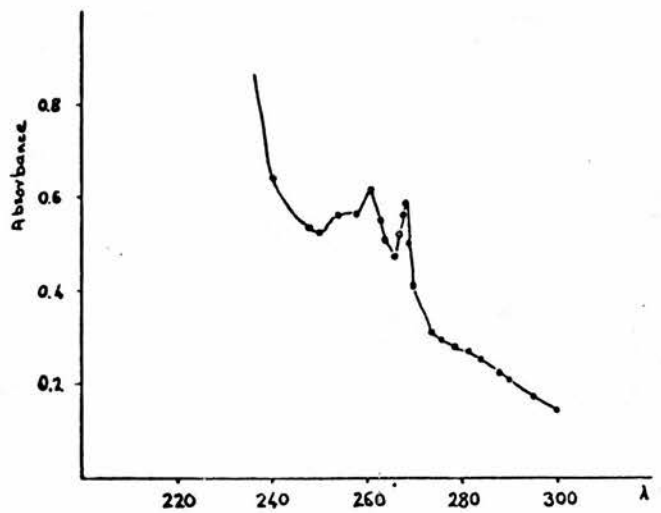
B. After pepsin- trypsin.

Fig 32e Cartilage from Squalus acanthias.

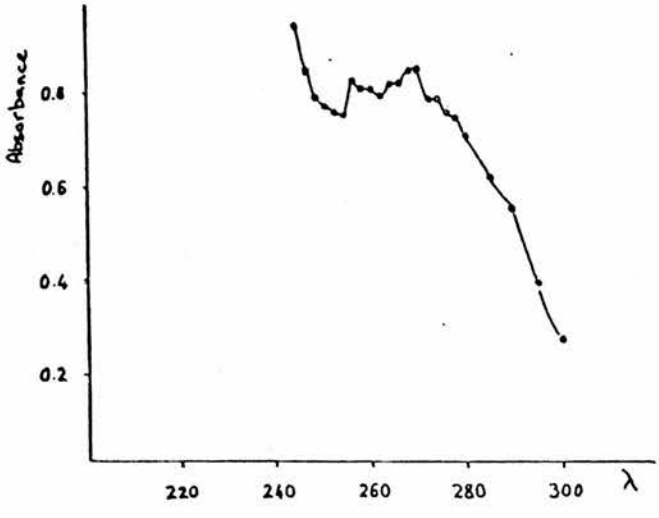
A. Collagenase digest autoclaved solution.

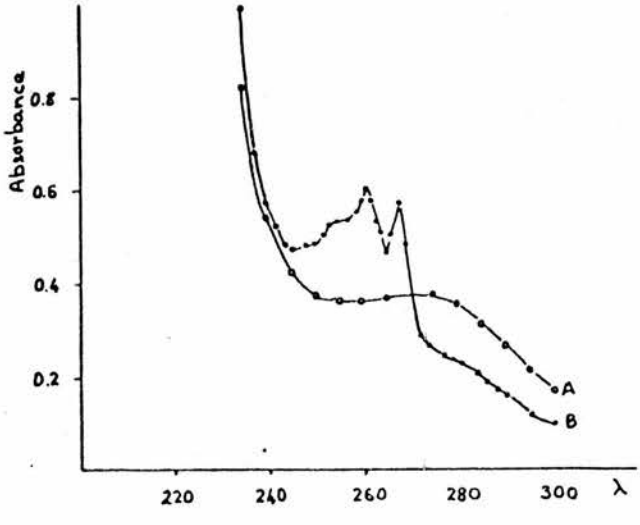
B. Collagenase digest heated in acid.


Absorbance
1.5
1.0
0.5
A
B
220
240
260
280
300
λ


Absorbance
0.8
0.6
0.4
0.2
220
240
260
280
300
λ

Figs. 32a&b


Absorbance
0.8
0.6
0.4
0.2
220
240
260
280
300
λ


Absorbance
0.8
0.6
0.4
0.2
220
240
260
280
300
λ
A
B

Figs. 32c&d

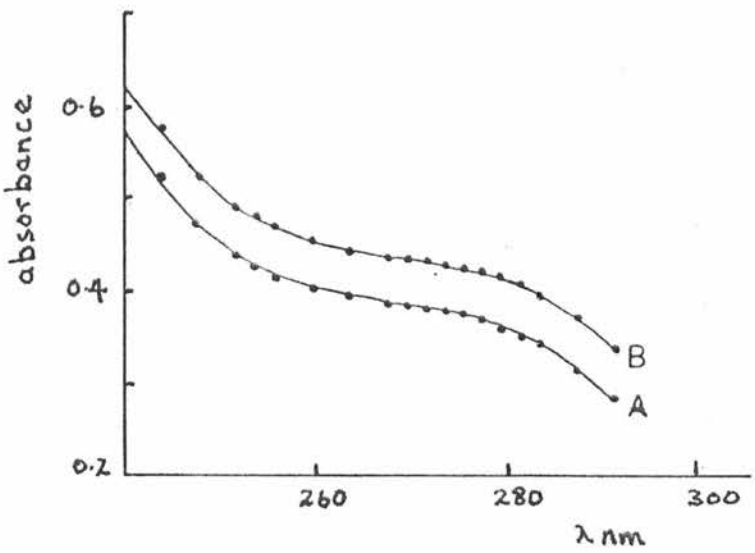



Fig. 32e

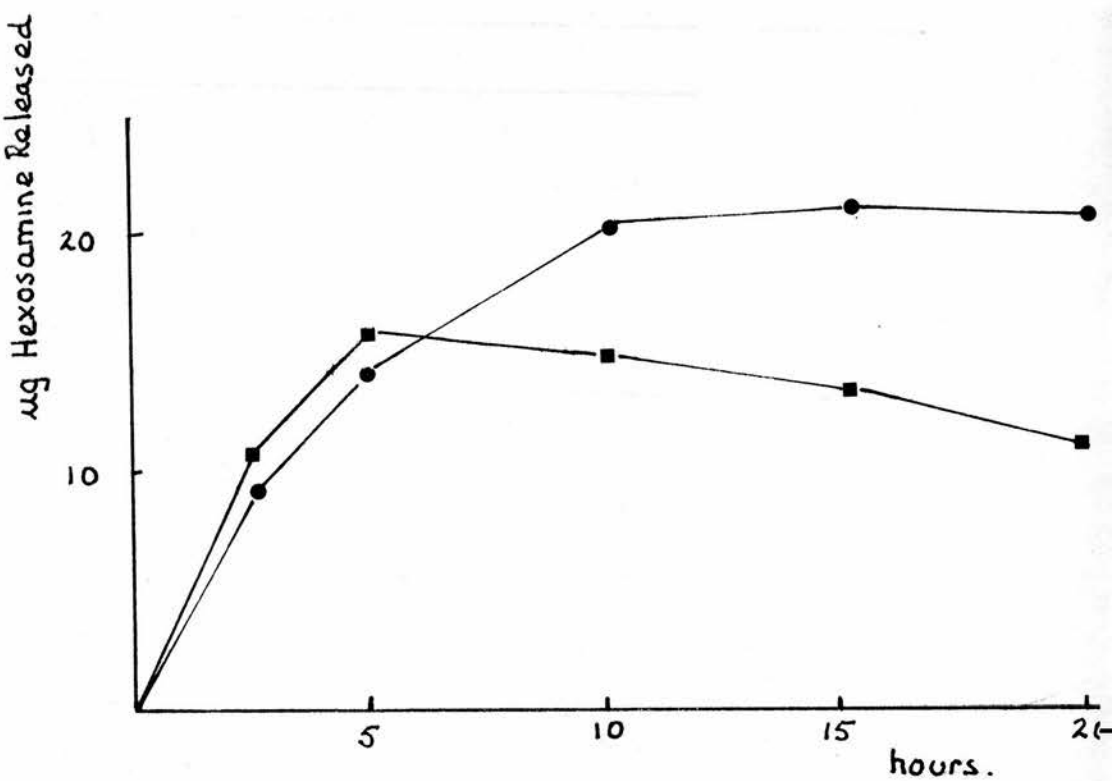



Fig.33

Figure 33 Release of hexosamine from Lorenzini glycopeptide following acid hydrolysis.

Samples of Sephadex G100 purified glycopeptide from Squalus acanthias ( 20 mg dissolved in water) were made 2M and 4M in HCl, and hydrolysed for varying periods at 105°C.

Aliquots were removed for the assay of hexosamine by the method of Gatt & Berman (1966), at various points in time.

2M HCl ● ● ●
4M HCl ■ ■ ■

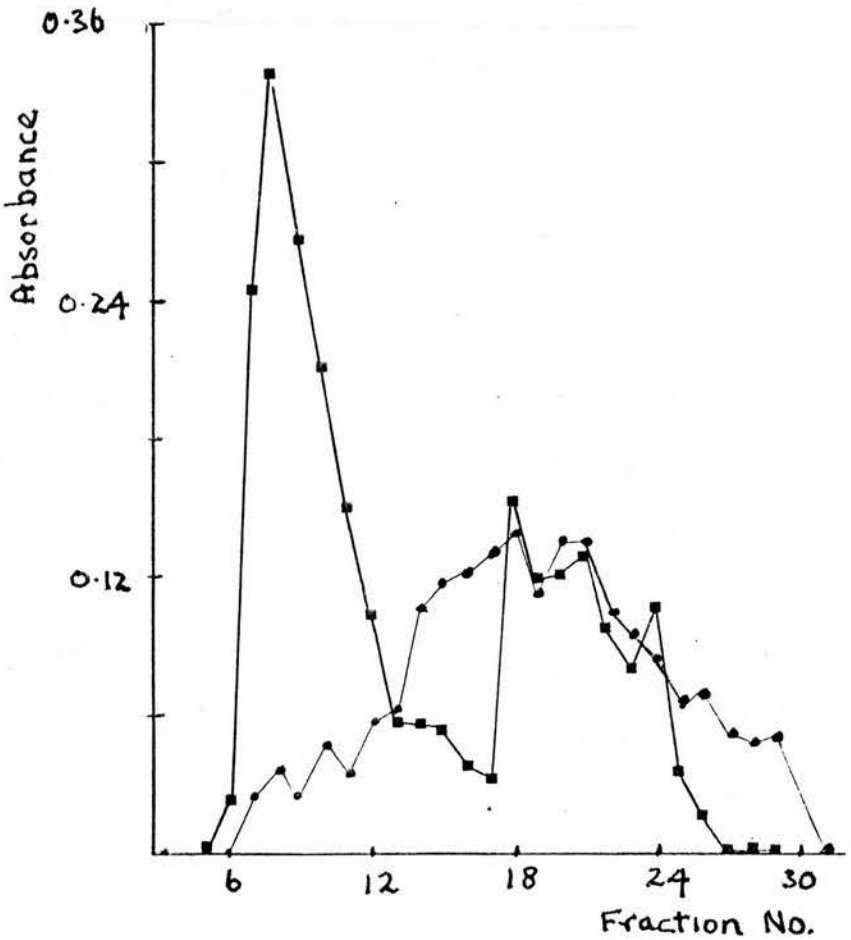



Fig 34

Figure 34 Fractionation of a papain digest of Galeorhinus galeus Lorenzini jelly on columns of Sephadex G150.

The column (42 x 5 cm) was packed in 1% NaCl, and 250 mg of crude digest freeze dried powder was applied dissolved in 20 ml of 1% NaCl. Elution was with the same solution, and 25 ml fractions were collected. Aliquots were analysed for carbohhdrate at 490 nm by the phenol/$H_2SO_4$ method and for protein using the method of Bradford (1976).

Phenol / $H_2SO_4$ 490 nm ■ ■ ■ ■

Coomassie Blue 595 nm ● ● ● ●

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# The 'Lorenzan Sulphates'

## A NEW GROUP OF VERTEBRATE MUCOPOLYSACCHARIDES

BY J. DOYLE
*Marine Station, Millport, Isle of Cumbrae, Scotland*

(*Received* 15 *August* 1966)

1. The mucopolysaccharide from the glands of Lorenzini of *Squalus acanthias* has been isolated and purified. 2. The material is a sulphated polysaccharide containing glucosamine, galactosamine, galactose and traces of an uronic acid. 3. This vertebrate polysaccharide is unusual in appearing to have both amino sugars present in the molecule. The amino sugars are *N*-acetylated. 4. This molecule is one of a group of related mucopolysaccharides which vary in composition with the animal species. The generic name 'lorenzan sulphates' is suggested to describe these substances.

The organs of Lorenzini are specialized sense organs which occur in elasmobranch fishes. In the shark-like fishes, they are located entirely in the head regions; in the rays, or skate-like fishes, the ducts can lead over the entire body surface to end in the pores at the trailing edge, an arrangement undoubtedly suited to the geometry and behaviour of the animal. Groups of small ampullae are served by branches of the facial nerve; from the nerve end the ampullae extend into tubes, often many centimetres in length, that end in a pore on the skin surface quite visible to the naked eye and sometimes more than a millimetre in diameter. The ampullae and the tubes are filled with a hyaline jelly which varies in 'stiffness' from species to species. This jelly is in contact with the sea water at the pore, and, at the other end, with the nerve end. The function of this organ is still uncertain, but the arrangement is extremely sensitive to a wide variety of artificially applied stimuli. An understanding of the chemical nature of this jelly might conceivably help in appreciating the physiological role of the system.

Jensen (1956) made a brief study, and found that the jelly contained mucopolysaccharide material, which he concluded, from its behaviour towards hyaluronidases, was a mixture of hyaluronic acid and chondroitin sulphates. Doyle (1963) examined the material from *Squalus acanthias* and performed chemical analyses which contradicted these conclusions, and showed galactose, glucosamine and galactosamine to be present. The small amount of uronic acid found did not agree with the presence of hyaluronic acid, and was not stoicheiometric with the amount of galactosamine. It was decided to extend these investigations by isolating the carbohydrate material and examining its properties. This paper is the first account of this work.

## MATERIALS AND METHODS

*Collection of jelly.* All material was from *Squalus acanthias*. Freshly caught specimens from the Clyde sea area were used, and the jelly was expressed from the pores in the head by pressure with a heavy spatula on the skin surface. About 1g. of wet jelly per adult animal can be collected this way. This represents a small percentage of the material present.

*Analysis of the crude jelly.* Since all pooled samples of jelly from *Squalus acanthias* showed an amino sugar ratio (glucosamine:galactosamine) 4:1, the possibility that this might be a statistical artifact was examined by determining the ratio for samples from four individual fish.

*Preparation of the polysaccharide.* A 92g. sample of fresh jelly in 1000 ml. of pH 6·5 buffer was digested with papain at 65° for 24hr. in the presence of activators, as recommended by Scott (1960). The digest was concentrated in a rotating evaporator at 50° to 100ml., filtered through Whatman no. 541 paper and purified on columns of Sephadex G-50. Columns of the Sephadex G-50 were equilibrated with water. Portions (5ml.) of the enzyme digest were added and allowed to drain into the column. This was eluted with water and 4·0ml. fractions were collected. Portions (0·1ml.) were removed for analysis. These were examined for carbohydrates by the anthrone reaction, for metachromasia with Azur A at pH2, and for $Cl^-$ ion (Fig. 1). The anthrone-positive component paralleled that showing metachromasia. The protein components were analysed by the method of Lowry, Rosebrough, Farr & Randall (1951). The anthrone-positive fractions (A) were pooled, concentrated and the crude material was precipitated by adding 5vol. of ethanol and leaving for 48hr. at 5°.

*Further purification.* Portions were treated by Sevag's method to remove residual protein, and then by treatment

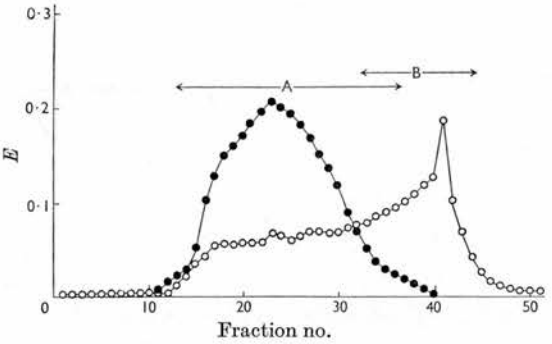


Fig. 1. Separation of major components of papain digest of Lorenzini jelly. Column, Sephadex G-50, 30cm.×2·5cm.; eluent, water. Fraction volume, 4ml. A, Metachromatic zone; B, $Cl^-$ ion. ●, Anthrone analysis; ○, protein.

on a column of DEAE-cellulose. DEAE-cellulose columns (2cm.×20cm.) in the chloride form were used. Columns were packed in 0·1M-NaCl. Portions estimated to contain 15mg. of crude polysaccharide were added to the column and washed in with 5ml. of water. The column was then washed with 100ml. of 0·1M-NaCl. The metachromatic material appearing with a further 100ml. volume of 1·0M-NaCl was concentrated at 50° *in vacuo* to 10ml. and the chloride removed by treatment on Sephadex G-50 as before.

*Chemical analyses.* Analysis of individual amino sugars was by Gardell's (1953) method, and total hexosamine analysis was performed by the technique of Boas (1953). Sulphate analysis (as total oxidizable sulphur) was by the method of Jones & Letham (1954). Nitrogen analysis was by the Kjeldahl method, with a Markham-type still. Neutral hexose was assayed by the anthrone method (Trevelyan & Harrison, 1952). Acetyl-group analysis was by the method of Ludowieg & Dorfman (1960). Uronic acid analysis was by the method of Bitter & Muir (1962).

*Paper electrophoresis.* This was carried out on 2·5cm.-wide strips of filter paper (Whatman no. 3) in 0·1M-veronal buffer, pH8·6, for 90min. at 15v/cm. Strips were dried and then stained in 1% Azur A in 0·01N-HCl. Excess of dye was washed off with dilute acetic acid. Ox-nasal-septum chondroitin sulphate (a mixture of the isomers) was run on parallel strips simultaneously as controls. Mixed solutions of the chondroitin sulphate and the Lorenzini-jelly polysaccharide were run in addition. For preparative purposes 30 runs were performed with the purified polysaccharide, five strips being used per run, and the position of the metachromatic bands was located by staining the outer millimetre of the two outer strips. The areas corresponding to the stained zone were cut out, pooled with all the other pieces and eluted with water, and the extracts were then dialysed against distilled water. The solution was then made 4·0N with respect to HCl, and hydrolysed for 14hr. at 105°. The evaporated solution was then subjected to fractionation and analysis for amino sugars according to the method of Gardell (1953).

*Fractional precipitation of the polysaccharide.* The technique of fractional precipitation of the calcium salts of chondroitin sulphate and keratosulphate as developed by Meyer, Linker, Davidson & Weissmann (1953) was used. The free acid (30mg.) was prepared by passing the polysaccharide through a column (2cm.×20cm.) of Zeo-Karb 225 cation-exchange resin in the $H^+$ form, washing with 50ml. of water. This was then concentrated to a volume of 4·0ml., and adjusted to an acetic acid concentration of 0·5N, and calcium acetate added to make a 5% concentration. Ethanol was added with stirring to various known concentrations, and the material left in this condition for 24hr. at 4° in between each addition. Any precipitated material was spun off and retained for analysis before further adjustments to the ethanol concentration were made.

*Paper chromatography.* The polysaccharide material was hydrolysed for 5hr. in N-$H_2SO_4$ at 105° in a sealed tube. Barium carbonate was added to neutrality, and the filtered solution concentrated and added to a small (2cm.×20cm.) column of Zeo-Karb 225 cation-exchanger. The acid and neutral sugar components were eluted with water. Descending chromatography was used with ethyl acetate–propan-1-ol–water (7:1:2, by vol.) and butanol–pyridine–water (5:3:2, by vol.) as the solvent systems. Portions of neutralized hydrolysate untreated by the resin technique were also used to identify the amino sugars by the method of Stoffyn & Jeanloz (1954), with the butanol–pyridine–water system.

*Identification of neutral sugars.* To confirm the chromatographic findings, attempts were made to form a derivative. The hydrolysate was freed from amino sugars by passing the neutral solution through a 2cm.×20cm. column of Zeo-Karb 225 ($H^+$ form) and washing the column with 50ml. of water; the total eluate was then concentrated at 50° *in vacuo* to near-dryness and taken up in 1·0ml. of water. A portion equivalent to 25mg. of purified polysaccharide was used. A portion (0·5ml.) of aqueous solution of the neutral sugar was mixed with 0·5ml. of ethanol, 0·05ml. of α-methylphenylhydrazine and 0·05ml. of 50% acetic acid. The solution was kept at 37° for 6hr. and then at 0° overnight. The crystals formed were washed with absolute ethanol and ether and dried in air. Standard and mixed melting points were made on the preparation and on authentic galactose α-methylphenylhydrazone prepared in the same way.

*Treatment with hyaluronidase.* Purified polysaccharide (15mg.) was incubated for 5 days at 37° in the presence of toluene, with 2·5mg. of testicular hyaluronidase (British Drug Houses Ltd., Poole, Dorset) in 4ml. of acetate buffer, pH6·5, made 0·15M with respect to NaCl. The experiment was performed twice, at different times and with different polysaccharide preparations. The effects of the enzyme on the amino sugar ratio were examined by separating the small-molecular material from the large on a column (22cm.×1·7cm.) of Sephadex G-75. The sample was applied and the column eluted with water. Fractions (1ml.) were collected, and the eluate was examined by the Molisch test, and by checking for metachromasia by spotting with Azur A in 0·01N-HCl on a white glazed porcelain tile. The metachromatic fractions were pooled in two lots arbitrarily divided for analysis. Fractions 6–16, and fractions 17–28, were taken for quantitative amino sugar analysis by Gardell's (1953) method. The small-molecular material, judged by chloride tests, started at fraction 30.

Table 1. *Hexosamine analysis (Gardell, 1953) on native Lorenzini jelly and on preparations*

Figures are expressed as a percentage of the total hexosamine.

| | | Glucosamine | Galactosamine |
|---|---|---|---|
| (A) Crude jelly from individual fish | (1) | 80·5 | 19·5 |
| | (2) | 78·8 | 21·2 |
| | (3) | 79·0 | 21·0 |
| | (4) | 79·7 | 20·3 |
| (B) Electrophoretically purified material | (1) | 78·8 | 21·2 |
| | (2) | 79·7 | 20·3 |

Table 2. *Chemical analyses of purified Lorenzini-jelly polysaccharide preparation (LS/2)*

Figures are for the sodium salt.

| | Percentage | Molar ratio |
|---|---|---|
| Galactose | 30·0 | 1·0 |
| Uronic acid | 2·85 | 0·08 |
| Total hexosamine | 28·6 | 0·96 |
| Acetyl | 7·4 | 1·03 |
| Sulphate | 14·5 | 0·91 |

*Mild alkali treatment.* Purified material (10mg.) was dissolved in 0·1N-NaOH, then heated at 40° for 1hr. The solution was neutralized and dialysed against several changes of veronal buffer, and subjected to paper electrophoresis as already described, ox-nasal-septum chondroitin sulphate and untreated polysaccharide being used as control strips during the run.

*Infrared spectrum.* Infrared-spectral analysis was performed by the courtesy of the Department of Chemistry, University of Birmingham. Polysaccharide (1·5mg.) was used with 300mg. of KBr in a KBr disk.

## RESULTS

The hexosamine analyses on crude jelly of the individual fish and for the various purifications and fractionations are shown in Table 1.

*Preparation of polysaccharide.* Crude jelly (92g.) gave 2·82g. of crude powder as the sodium salt. This crude material had a total hexosamine content of 23·9%. The original jelly had 10·5$\mu$g. of hexosamine/mg. wet wt. of jelly, the recovery thus being 70% at this stage. A further 0·51g. of material from the ethanolic solution was collected on standing for some weeks. This gives a yield of crude material of 83% in terms of the starting material. The nitrogen content of this material (designated LS/1) was 4·34%, and the further purification steps (Sevag's deproteinizing method), followed by purification on ion-exchange columns, reduced this nitrogen figure to 2·95%. This purified material was designated LS/2.

*Chemical analysis.* The analytical results for LS/2 are given in Table 2 together with the molar ratios relative to galactose. The figures are uncorrected for hydrolytic degradation.

The acetyl group is evidently as *N*-acetyl, presumably on the hexosamine, for the purified material gave a negative ninhydrin reaction, but after treatment with 0·1N-sulphuric acid for 1hr. at 100° the ninhydrin reaction was strongly positive.

*Electrophoresis.* Fig. 2 shows a metachromatic band travelling at about half the rate of mammalian chondroitin sulphate. Untreated (protein-bound) jelly material does not move from the origin. Mixtures of chondroitin sulphate and the purified preparation are readily separated.

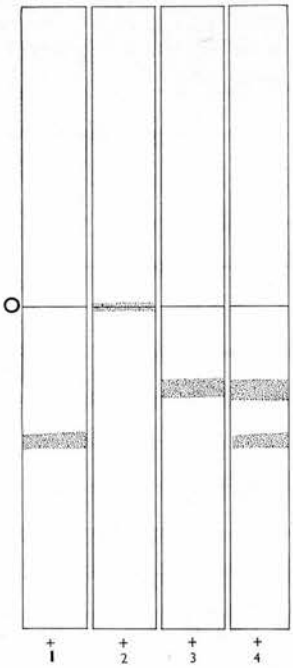


Fig. 2. Paper electrophoresis diagram of mucopolysaccharides from Lorenzini jelly. See the text for conditions. O, Origin. 1, Chondroitin sulphate. 2, Native Lorenzini jelly. 3, Purified lorenzan sulphate. 4, 1 + 3.

*Preparative electrophoresis.* The hexosamine ratio of the material eluted from the metachromatic bands after electrophoresis on duplicate sets of experiments was the same as that in the crude jelly and the purified preparation (Table 1).

Fig. 3 shows the results of analysis of the amino sugar analysis by the Gardell (1953) technique. The separation shown is typical of all such analyses reported.

*Ethanol precipitation.* The yields of calcium salts at various concentrations of ethanol, and hexosamine analysis of the fractions, are given in Table 3.

*Paper chromatography.* Galactose was the only neutral sugar found. The $R_{Glc}$ values were 0·87 in the ethyl acetate solvent system and 0·89 in the other. Trace amounts of uronic acids were seen, but no positive identification of the nature of the uronic acid was made. The amino sugar analysis gave $R_{Glc}$ 1·75 for lyxose (galactosamine) and 1·31 for arabinose (glucosamine).

*α-Methylphenylhydrazone.* The chromatographic evidence was confirmed by the formation of the α-methylphenylhydrazone derivative. This had m.p. 179–180° (uncorrected) and gave mixed m.p. 179–181° with the authentic galactose derivative.

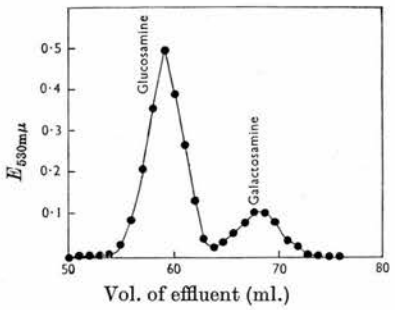


Fig. 3. Typical chromatographic analysis of the amino sugars of purified mucopolysaccharide from Lorenzini jelly of *Squalus acanthias*. The analysis is of the ethanolic (50%) precipitate of the calcium salt; 2·35mg. of the calcium salt was applied to the column. The method is according to Gardell (1953).

*Hyaluronidase treatment.* The effect of this enzyme on the polymer is not great, inasmuch as about 90% of the macromolecule was recovered as such after treatment. Its behaviour on gel-filtration columns, and its metachromasia, were consistent with a sulphated polysaccharide. The amino sugar ratios after enzyme treatment and fractionation on the Sephadex column were slightly changed from that of the purified material (Table 4).

*Alkali treatment.* Electrophoresis results were as for untreated material. No change in electrophoretic mobility was observed after this treatment,

Table 4. *Effects of hyaluronidase on the amino sugar ratio of purified Lorenzini-jelly mucopolysaccharide*

The material after enzyme treatment was separated on Sephadex G-75 columns from low-molecular-weight material, and the fractions of macromolecular material were arbitrarily separated into two groups on the basis of their elution from the Sephadex column. Results of duplicate experiments are reported.

| | Total hexosamine (%) | |
|---|---|---|
| | Glucosamine | Galactosamine |
| Expt. 1 | | |
| Fractions 6–16 | 74·4 | 25·6 |
| Fractions 17–28 | 82·5 | 17·5 |
| Expt. 2 | | |
| Fractions 6–16 | 74·6 | 25·4 |
| Fractions 17–28 | 83·3 | 16·7 |

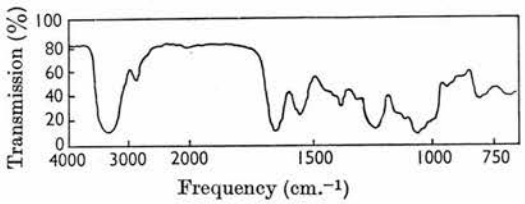


Fig. 4. Infrared spectrum of mucopolysaccharide from Lorenzini jelly of *Squalus acanthias*.

Table 3. *Yields of calcium salts mucopolysaccharide, at various concentrations of ethanol, and hexosamine analysis of the fractions*

| Final concn. of ethanol (%) | Yield of calcium salt (mg.) | Total hexosamine (%) | |
|---|---|---|---|
| | | Glucosamine | Galactosamine |
| 25 | — | — | — |
| 35 | — | — | — |
| 50 | 17·5 | 77·9 | 22·1 |
| 66 | 10·9 | 82·4 | 17·6 |
| 80 | — | — | — |

and only one metachromatically staining band was found.

*Infrared analysis.* The 1240cm.$^{-1}$ (S=O stretching) characteristic of sulphate is present (Fig. 4). At 1550 and 1640cm.$^{-1}$ there are strong absorptions attributable to the presence of *N*-acetyl residues. There is not much evidence for either ionized or non-ionized carboxylic acid functions which give absorptions at 1736 and 1230cm.$^{-1}$ ($CO_2H$) and 1410cm.$^{-1}$ ($CO_2^-$).

## DISCUSSION

The constancy of composition, particularly as it is expressed in the amino sugar ratios, both of pooled samples and from some individual animals, provides grounds for suspecting the existence of a true chemical compound, and not a mixture of, say, a chondroitin sulphate and keratan sulphate. In view of the very low uronic acid figures, it is difficult to account for the results in this way. The presence of uronic acid was in doubt for some time during this study. However, its presence has been repeatedly indicated by the Bitter & Muir (1962) technique, and sample blanks were used to compensate for non-specific colour formed by charring of the neutral sugars. The paper-chromatographic evidence for the presence of uronic acids would not be sufficient alone. The infrared-spectral analysis shows little or no sign of uronic acid. The galactose, total hexosamine, acetyl and sulphate appear in a molar ratio close to unity. The behaviour of the substance on gel-filtration columns, and to dialysis, and its metachromasia indicate that the molecule is large; no measurements of size have yet been made, although they would clearly be desirable. The nitrogen figures for the purified material (LS/2) indicate that the material is not entirely free from residues of protein material. It is doubtful, in view of recent structural studies on the linkage of protein to polysaccharide, whether any mild proteolytic preparative technique can free the polysaccharide entirely from amino acid residues. This is a consequence of mild preparative techniques. The evidence from this study can be accounted for by the hypothesis of a single molecular compound involving both amino sugars. Electrophoretic evidence is clear-cut, and even treatment with sodium hydroxide failed to demonstrate a separation of a chondroitin sulphate and a keratan sulphate. The finding of both amino sugars in the original ratio in this electrophoresis band is difficult to account for on the basis of any known mixture. The results of the calcium salt fractionation confirm this; no precipitation occurred below 50% ethanol concentration, a range in which one would expect chondroitin sulphate to be precipitated. Other ways of accounting for the presence of galactosamine and for the constancy of composition seem less probable.

The nature of the neutral sugar as galactose is in no doubt, and this establishes this molecule as a closer relative of keratan sulphate than of the chondroitin sulphates. It is noteworthy that the first recorded description of what was clearly keratan sulphate was also in tissue from an elasmobranch fish (Hisamura, 1938).

The behaviour of the purified material towards testicular hyaluronidase is interesting. Jensen (1956) observed changes in viscosity with testicular hyaluronidase, and also with bacterial hyaluronidase. From this, he concluded that the jelly was a mixture of chondroitin sulphate and hyaluronic acid. This, from the analytical findings reported earlier (Doyle, 1963) and in this paper, is wrong. Indeed, the enzymic evidence reported in this study shows the molecule to be to a large extent resistant to the testicular enzyme. A small variation in the amino sugar ratio was observed, the relatively larger molecules (those fractions eluted first from the gel-filtration column) being slightly enriched in galactosamine with respect to the low-molecular-weight material. This was not observed with material untreated by the enzyme. The change is not great, however, but may be of interest in subsequent structural studies. The division into two groups was quite arbitrary, as the anthrone analysis of the column effluent gave no indication of more than one peak. It is possible that although no great chemical degradative changes have taken place, yet some depolymerization may have occurred. This would account for Jensen's (1956) findings. Keratan sulphate itself, which would appear to be the closest known relative of this molecular species, is unaffected by testicular hyaluronidase.

The infrared analysis supports generally the analytical findings and agrees in assigning the position of acetyl groups to the amino group of the amino sugar. It also underlines the differences between this material and known mucopolysaccharides.

The function of this jelly in the sense organ is uncertain; so indeed to some extent is the function of the organ itself. This lack of understanding, however, is no reflection on the importance of the organ to the animal. The nature, size and arrangement of this sensory apparatus point to its being important, and it is receiving the attention of physiologists. It would be possible to suppose that the mucopolysaccharide might bind pharmacologically active bases and release them to the nerve ending on changes of temperature or salinity, and thus account for some of the physiological findings mechanistically. The proposed name 'lorenzan sulphates' (followed by the species of the

animal) is formed from the name of the Italian biologist who first described these organs, and the name ending is made consistent with modern terminological practice. Thus the compound described in this preliminary study would be 'lorenzan sulphate (*Squalus acanthias*)'.

I acknowledge gratefully the help of Dr Mervyn How, Department of Chemistry, University of Birmingham, with the infrared studies. I am indebted to Miss Elspeth J. Kitchin for skilled technical assistance.

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# THE LORENZAN SULPHATES: A COMPARATIVE STUDY

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**Abstract**—1. The jelly of the ampullae of Lorenzini, a sense organ occurring in some groups of fish, contains a mucopolysaccharide–protein complex whose carbohydrate moiety varies from species to species.

2. This group of carbohydrates, termed the Lorenzan sulphates, have the same constituents present in different proportions, viz. glucosamine, galactosamine, galactose, sulphate and traces of uronic acid.

3. The analytical findings do not allow of interpretation in terms of known vertebrate mucopolysaccharide mixtures.

## INTRODUCTION

THE AMPULLAE of Lorenzini were first described in detail by Lorenzini (1678). They occur in all true cartilaginous fish and also in the siluroid, *Plotosus*. The organ consists of an ampulla connected to a branch of the facial nerve, and which is also connected to the environment of the fish by a tube ending in a pore on the skin surface. Both the ampulla and the tube are filled with a jelly-like material. The system has the same histological origin as the lateral line system, but is functionally a different organ (Dijkgraaf & Kalmijn, 1963). Recently, Kalmijn (1966) demonstrated elegantly that the function of the ampullary system is to detect motion in other animals by sensitive electro-perception of their spread muscle potentials. His experiments explain aspects of shark behaviour not previously understood; divers have described the extraordinary speed with which sharks can appear on the scene when a fish is seriously disturbed or wounded (Eibl-Eibesfeldt & Hass, 1959).

The chemical nature of the jelly which fills the organ has been studied (Doyle, 1963, 1967) with a view to elucidating the physiological mechanism involved. From *Squalus acanthias* a mucopolysaccharide was purified containing glucosamine, galactosamine and galactose, and which was *N*-acetylated and sulphated. The present study shows that this substance represents one of a group in which considerable variation occurs between the species.

## MATERIALS AND METHODS

### *Biological material*

All fish were collected from various sea areas around Britain. For the most part, freshly trawled animals were used, and the jelly expressed from the pores by pressure with a lever. Except when the fish was extremely large or relatively

rare, the samples of jelly were pooled from numbers of animals of the same species. The native jelly was stored in sealed bottles at $-20°C$ until it was analysed. Some fish were obtained in a deep frozen state from commercial trawlers based in Aberdeen. Such fish were thawed before removal of the jelly. In larger sharks, jelly was removed by dissection of the head because it is not possible to exert enough pressure on these from the skin.

*Preparation of the jelly material for analysis*

In all cases the native jelly was weighed and dissolved in distilled water by shaking, to produce solutions of suitable concentration for the analytical procedures. In the case of those animals quoted herein where i.r. and metachromatic spectra were measured, purified preparations were made by digestion with papain as reported previously (Doyle, 1967). For purposes of paper chromatography, solutions of the jelly were dialysed against distilled water to remove salts and urea. It follows that these results refer to macromolecular components.

*Chemical analysis methods*

*Hexosamine.* Aliquots of solutions of jelly were hydrolysed in 3·0 N HCl for 12 hr at 100°C. Total hexosamine was estimated according to the method of Boas (1953). The ratio of hexosamines was determined by Gardell's (1953) method.

*Hexose.* Unhydrolysed solutions were used, and hexose determined according to Trevelyan & Harrison (1952). D-Galactose (BDH) was used as a standard.

*Nitrogen.* Nitrogen was analysed on weighed pieces of jelly using a Markham-type still by the Kjeldahl method.

*Uronic acid.* This was analysed according to Bitter & Muir (1962). Sample blanks were used to offset non-specific colour from neutral sugars.

*Paper chromatography*

The dialysed solution of native jelly was hydrolysed in N $H_2SO_4$ for 5 hr at 100°C. Solid $BaCO_3$ was added to neutrality and the solution filtered and concentrated. Amino sugars were removed on a column of Zeokarb 225 cation exchanger, $15 \times 1{\cdot}5$ cm. Neutral sugars were eluted with water and the resulting solution concentrated for paper chromatography. Amino sugars were eluted with N $H_2SO_4$ which was removed as before by $BaCO_3$. The solvent systems used were ethyl acetate : propan-1-ol : water (7 : 1 : 2, by vol.) and butanol–pyridine–water (5 : 3 : 2, by vol.). The amino sugars were detected as pentoses after reaction with ninhydrin (Stoffyn & Jeanloz, 1954).

*Infra-red analysis*

Purified polysaccharide powder (1·5 mg) as the sodium salt was used with 300 mg KBr in a KBr disc.

*Metachromasia*

Metachromasia was detected qualitatively on a porcelein spotting tile using Azur A in 0·01 N HCl. The spectral curves of metachromasia were measured on

solutions of sodium salts of purified polysaccharide. Polysaccharide (2 mg) was mixed with 1 mg Azur A in a volume of 20 ml which was adjusted to pH 3·0 by HCl.

*Sulphate analysis*

Sulphate was analysed on purified polysaccharide preparations only from *Galeorhinus galeus* by the method of Jones & Letham (1954).

## RESULTS AND DISCUSSION

The chemical analytical findings are summarized in Table 1. The results, quoted on a wet weight basis, are meaningful only in relation to the other components in material from the same animal. The jelly itself varies considerably in stiffness or rigidity between the species, and this appears to be a concentration effect. The material from the larger sharks, e.g. *Cetorhinus maximus*, has a relatively rigid jelly which it possibly requires, as the pore size in these large animals is also large. In this species the pores are elliptical and about 3–4 mm long. A small shark or dogfish, e.g. *Squalus acanthias*, has a more fluid jelly and a round pore about 0·5 mm dia. The results quoted for purified material (Doyle, 1967) from *Squalus acanthias* showed a 4 : 1 ratio for glucosamine : galactosamine. The presence of so much galactosamine which could not be separated from glucosamine gave grounds for believing that this was not keratosulphate but some new compound. Since that work was started, Seno *et al.* (1965) have reported that keratosulphates contain some galactosamine, and Meyer (personal communication) and that the galactosamyl group provides the linkage between the protein and the carbohydrate chains in keratosulphate. However, the amounts of galactosamine are relatively small, whilst in the lorenzan sulphates, some have approximately an equimolar ratio between the amino sugars, and in the two species of holocephali examined, the galactosamine predominates over the glucosamine (Fig. 1).

The other point of interest in the results quoted here is that the galactose is not always in approximately 1 : 1 molar ratio with total hexosamine. Indeed, in *Cetorhinus maximus*, there is more than twice as much galactose as amino sugar—a situation which is reversed in *Chimaera monstrosa*. These deviations must be regarded as well outside the limits of error for the methods. A further point which differentiates the group from known vertebrate mucopolysaccharides is that unsulphated material exists. In material from *Galeorhinus galeus*, sulphate analysis indicated no sulphate to be present. This accorded with the information from the i.r. spectrum (Fig. 2).

*Infra-red analysis*

The infra-red spectrum of purified material from *Squalus acanthias* shows a strong absorption band at 1240 $cm^{-1}$ (S=O stretching), and other absorptions in the 800–950 $cm^{-1}$ region, characteristic of sulphate. These absorptions are absent in the spectrum of purified material from *Galeorhinus galeus*. In other respects, the two spectra show comparable features. They have strong absorptions in the

TABLE 1

| | Nitrogen (μg/mg) | Total hexosamine (μg/mg) | % Total Glucosamine | % Total Galacto-samine | Uronic acid (mg/μg) | Hexose (μg/mg) | Meta-chromasia at pH 2·0 | | Type |
|---|---|---|---|---|---|---|---|---|---|
| *Carcharinus glaucus* | 6·38 | 9·6 | 60·0 | 40·0 | 0·55 | 9·82 | + | Large shark | Shark-like |
| *Cetorhinus maximus* | 13·8 | 13·8 | 57·1 | 42·9 | 0·58 | 31·5 | + | | |
| *Galeorhinus galeus* | 6·2 | 10·9 | 46·5 | 53·5 | 0·43 | 12·2 | − | | |
| *Galeus melanistomus* | 3·48 | 6·17 | 84·5 | 15·5 | 0·36 | 7·24 | + | | |
| *Scylliorhinus canicula* | 1·81 | 7·8 | 71·0 | 29·0 | 0·42 | 10·7 | + | | |
| *Scylliorhinus stellaris* | 4·82 | 15·12 | 65·6 | 34·4 | 0·87 | 17·9 | + | | |
| *Squalus acanthias* | 7·58 | 10·5 | 80·0 | 20·0 | 0·58 | 11·6 | + | | |
| *Raja batis* | 6·0 | 3·7 | 79·0 | 21·0 | 0·93 | 4·75 | + | Large ray | Ray-like |
| *Raja brachyurus* | 2·75 | 4·63 | 87·0 | 13·0 | 0·52 | 6·3 | + | | |
| *Raja clavata* | 3·03 | 5·93 | 80·6 | 19·4 | 0·50 | 5·4 | + | | |
| *Raja microcellata* | 3·65 | 4·4 | 76·1 | 23·9 | 0·56 | 4·92 | + | | |
| *Raja montagui* | 2·80 | 6·2 | 83·3 | 16·7 | 0·76 | 8·63 | + | | |
| *Raja naevus* | 2·45 | 5·7 | 87·6 | 12·4 | 0·40 | 5·92 | + | | |
| *Torpedo nobiliana* | 6·07 | 10·3 | 58·7 | 41·3 | 0·45 | 10·0 | + | | |
| *Hydrolagus affinis* | 2·78 | 2·44 | 11·19 | 88·81 | 0·15 | 2·29 | + | | Holocephalic |
| *Chimera monstrosa* | 6·81 | 3·2 | 20·5 | 79·5 | 0·16 | 1·50 | + | | |

1550 and 1640 $cm^{-1}$ region, attributable to *N*-acetyl residues. Ionized and non-ionized carboxylic acid functions are not in evidence, as judged by the weakness of

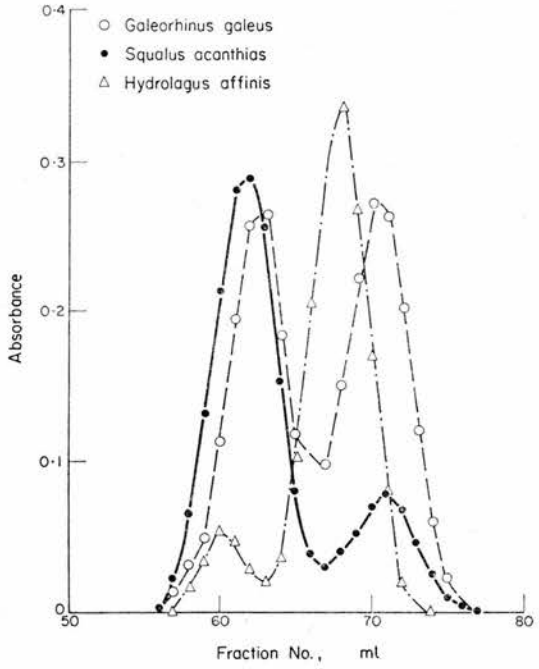


FIG. 1. Amino sugar analysis of Lorenzini jellies according to Gardell's method (1953). The first peak in each case represents glucosamine.

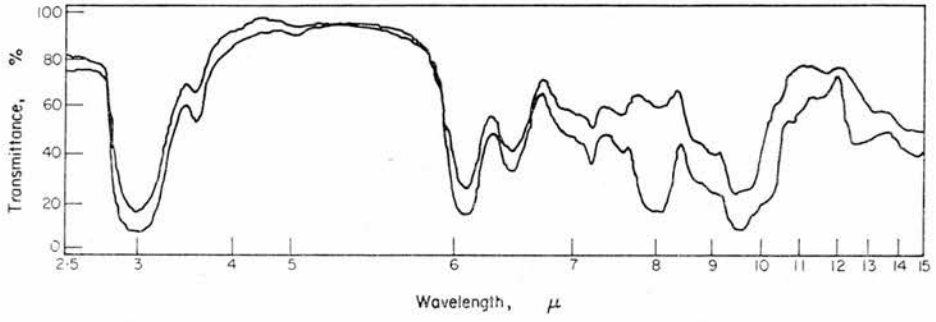


FIG. 2. Infra-red spectrum of papain digest preparations of Lorenzan sulphates of (1) *Galeorhinus galeus* (upper graph); (2) *Squalus acanthias* (lower graph).

absorptions at 1736 and 1230 $cm^{-1}$ (COOH), and 1610 and 1410 $cm^{-1}$ ($COO^-$). Small amounts of uronic acid have in fact been detected by the Bitter & Muir analytical method in all species examined. The evidence for uronic acid is not supported by the i.r. information, whilst the paper chromatographic evidence on this point was not clear.

*Metachromasia*

A comparison between the spectrum of mixtures of Azur A and polysaccharides is shown for *Galeorhinus galeuss* and *Squalus acanthias* preparations. The bathochromatic shift in the absorption maximum wavelength is evidenced in *Squalus acanthias* only. All the material from the other species examined proved to be metachromatic at acid pH, i.e. the material is sulphated.

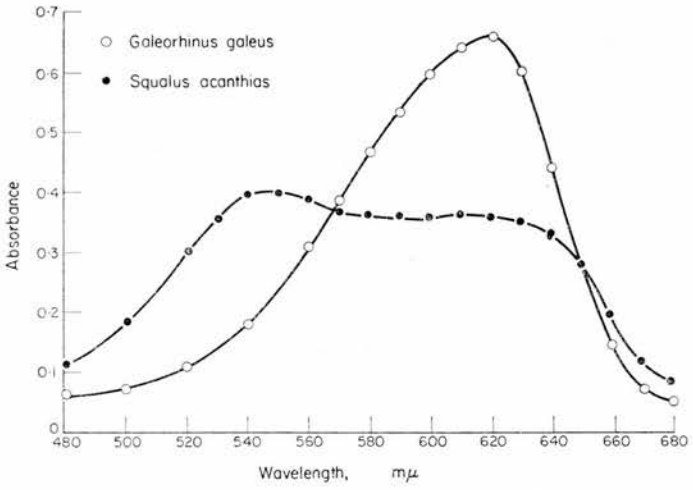


FIG. 3. Comparative metachromatic activity of purified Lorenzan sulphates on Azur A. For conditions, see text.

*Neutral sugars*

In all species, galactose was clearly identified as the only neutral sugar component.

## SUMMARY

A comparative analytical study of the lorenzan sulphates from a number of cartilaginous fish was performed. The polysaccharide component varies considerably in the ratio of the two amino sugars on a species basis. The amount of galactose is not always in 1 : 1 molar ratio with total hexosamine, and one case of an unsulphated polymer is reported.

*Acknowledgements*—I am indebted to Dr. Corner and Mr. Mattacola of the Plymouth Laboratory for help with some specimens, and to Dr. Bennet B. Ray, of the Marine Laboratory, Aberdeen, for apecimens and advice on sharks.

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# AGEING CHANGES IN CARTILAGE FROM *SQUALUS ACANTHIAS* L.

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(*Received* 3 *August* 1967)

**Abstract**—1. The ageing of cartilage in an elasmobranch fish has been studied and compared with the situation in mammals.

2. The ratio of keratosulphate to chondroitin sulphate in *Squalus acanthias* cartilage is roughly constant with age, unlike mammals.

3. The ash content of *Squalus* cartilage changes considerably with age.

## INTRODUCTION

AGEING changes in the mucopolysaccharide content of mammalian cartilage have been studied in many species since Shetlar & Masters (1955) examined the situation in human costal cartilage. Both uronic acid and hexosamine concentrations were found to decrease with age, but not in the same way, implying that not all of the changes took place in substances where hexosamine was bound to uronic acid. In 1958, Stidworthy *et al.* showed that galactosamine concentrations fell at the same rate as uronic acid concentrations. Kaplan & Meyer (1959), also using human costal cartilage, showed that if the ratio of keratosulphate to total mucopolysaccharide (i.e. the glucosamine-containing material) is plotted against age, a linear correlation was found. Anderson & Odell (1960) demonstrated the effect in rat cartilage. Doyle (unpublished work) found similar changes in rabbit cartilage. Szirmai & Doyle (unpublished work) found evidence of similar changes in the nasal septum cartilage of ox and of horse.

Because it might be of interest to extend these observations to a species other than a mammalian one, and also because ichthyologists cannot use scale or otolith measurements as an index of age in cartilaginous fish, as they can in teleosts, the present study was initiated on pectoral cartilage of *Squalus acanthias.*

## MATERIALS AND METHODS

All animals were trawled in the Clyde sea area except for the smallest (free-swimming) which were taken further north off the Scottish west coast. The fish were measured and weighed on arrival, and the entire pectoral girdle dissected out. The small morphologically distinct piece of cartilage containing calcified rings which occurs in all fish, irrespective of size, was discarded. Perichondrial tissue was also removed. Whole girdles, or, in the case of larger fish, symmetrical half-girdles, were taken and cut by hand into thin slices and dehydrated for several days

TABLE 1—CHEMICAL COMPOSITION OF *Squalus cartilage*

| | Sex | Length (cm) | Weight (g) | Dry wt. uronic acid (μg/mg) | Dry wt. total hexosamine (μg/mg) | Total hexosamine glucosamine (%) | Total hexosamine galactosamine (%) | Dry wt. ash (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | ♂ | 27·9 | 63·0 | 85·6 | 99·0 | 9·9 | 90·1 | — |
| 2 | ♂ | 28·7 | 84·0 | 74·6 | 79·6 | 9·6 | 90·4 | 11·8 |
| 3 | ♂ | 31·9 | 99·5 | 74·0 | 82·3 | 11·1 | 88·9 | 19·9 |
| 4 | ♀ | 32·6 | 139·9 | 81·7 | 87·5 | 8·4 | 91·6 | 19·2 |
| 5 | ♀ | 36·7 | 163·0 | 85·0 | 80·6 | 8·2 | 91·8 | 16·6 |
| 6 | ♂ | 38·6 | 195·7 | 75·6 | 76·5 | 7·8 | 92·2 | 21·3 |
| 7 | ♂ | 40·1 | 193·9 | 80·5 | 80·5 | 10·0 | 90·0 | 22·8 |
| 8 | ♂ | 40·5 | 220·1 | 82·6 | 79·5 | 10·1 | 89·9 | 28·6 |
| 9 | ♀ | 45·0 | 348·6 | 79·1 | 75·5 | 6·1 | 93·9 | 19·3 |
| 10 | ♂ | 45·5 | 404·4 | 84·3 | 78·0 | 7·5 | 92·5 | 29·6 |
| 11 | ♂ | 45·5 | 352·6 | 78·0 | 73·6 | 6·4 | 92·6 | 22·6 |
| 12 | ♀ | 47·5 | 423·6 | 85·0 | 79·6 | 6·8 | 93·2 | 19·8 |
| 13 | ♂ | 48·5 | 416·3 | 83·6 | 75·1 | 9·1 | 90·9 | 27·6 |
| 14 | ♀ | 50·0 | 451·4 | 82·4 | 77·8 | 7·2 | 92·8 | 26·1 |
| 15 | ♂ | 51·5 | 462·0 | 92·1 | 84·7 | 7·5 | 92·5 | 23·1 |
| 16 | ♀ | 51·5 | 544·4 | 89·4 | 86·3 | 9·8 | 90·2 | 25·4 |
| 17 | ♀ | 53·5 | 547·9 | 82·8 | 81·1 | 8·0 | 92·0 | 23·6 |
| 18 | ♀ | 54·5 | 633·6 | 87·8 | 85·0 | 8·3 | 91·7 | 23·4 |
| 19 | ♀ | 58·0 | 716·8 | 84·4 | 83·9 | 7·4 | 92·6 | 24·0 |
| 20 | ♀ | 58·0 | 675·9 | 80·6 | 77·6 | 8·1 | 91·9 | 26·6 |
| 21 | ♀ | 58·0 | 647·8 | 81·2 | 78·0 | 7·5 | 92·5 | 28·0 |
| 22 | ♂ | 58·0 | 637·8 | 87·8 | 85·0 | 9·1 | 90·9 | 26·1 |
| 23 | ♀ | 58·5 | 734·8 | 81·7 | 75·5 | 8·6 | 91·4 | 25·0 |
| 24 | ♀ | 60·5 | 767·7 | 82·6 | 77·0 | 8·6 | 91·4 | 29·5 |
| 25 | ♀ | 61·5 | 811·9 | 83·4 | 77·2 | 7·1 | 92·9 | 22·5 |
| 26 | ♀ | 62·5 | 860·4 | 79·1 | 74·8 | 6·2 | 93·8 | 29·1 |
| 27 | ♀ | 63·5 | 954·6 | 85·3 | 75·0 | 8·2 | 91·8 | 28·8 |
| 28 | ♀ | 66·0 | 1044·7 | 90·6 | 83·0 | 6·5 | 93·5 | 29·1 |
| 29 | ♂ | 66·0 | 1008·0 | 54·4 | 56·8 | 8·7 | 91·3 | 35·7 |
| 30 | ♀ | 66·5 | 1112·4 | 82·3 | 77·5 | 10·4 | 89·6 | 36·0 |
| 31 | ♂ | 68·0 | 1129·0 | 28·2 | 29·0 | 10·9 | 89·1 | 48·6 |
| 32 | ♀ | 70·0 | 1318·6 | 81·4 | 75·0 | 9·5 | 90·5 | 33·4 |
| 33 | ♀ | 70·0 | 1420·8 | 83·7 | 73·8 | 6·6 | 93·4 | 37·0 |
| 34 | ♂ | 71·7 | 1267·0 | 63·8 | 54·5 | 6·9 | 93·1 | 48·6 |
| 35 | ♀ | 72·5 | 1358·0 | 76·2 | 70·0 | 9·0 | 91·0 | 38·1 |
| 36 | ♂ | 74·0 | 1680·0 | 38·4 | 38·9 | 9·1 | 90·9 | 39·6 |
| 37 | ♀ | 75·0 | 1820·0 | 78·7 | 76·5 | 6·3 | 93·7 | 34·1 |
| 38 | ♂ | 77·5 | 1628·0 | 54·4 | 57·1 | 6·1 | 93·9 | 43·0 |
| 39 | ♀ | 87·5 | 2531·0 | 61·2 | 58·0 | 7·5 | 92·5 | 34·3 |
| 40 | ♀ | 87·5 | 2540·0 | 80·0 | 81·0 | 10·7 | 89·3 | 38·5 |
| 41 | ♀ | 97·0 | 4660·0 | 48·9 | 96·6 | 10·2 | 89·8 | 47·5 |
| 42 | ♀ | 98·0 | 4545·0 | 39·4 | 53·0 | 11·2 | 88·8 | 44·0 |
| 43 | ♀ | 98·0 | 4320·0 | 17·5 | 21·0 | 11·2 | 88·8 | 43·7 |

in acetone with frequent changes of solvent. This dry material was ground to a fine powder and dried in a desiccator for some weeks over $P_2O_5$. Analyses were performed on this dried powdered material after weighings indicated no further changes in mass.

*Analytical methods*

Uronic acid was estimated on the finely ground material by the method used by Shetlar & Masters (1955).

Total hexosamine was estimated according to Boas (1953) after hydrolysis in 4 N HCl for 14 hr at 105°C. The ratio of the two amino sugars was found by separation on ion exchange columns according to Gardell (1953).

Ash was determined by weight difference on heating to 500°C in an electric furnace to constant weight. A few drops of N $H_2SO_4$ were added previous to strong heating to convert any carbonates to sulphates.

## RESULTS

The analytical results are presented in Table 1.

Uronic acid concentrations seem approximately the same as hexosamine; the analytical results are consistent with those found for the amino sugar ratios; that is, about 10 per cent of the total mucopolysaccharide present is keratosulphate, which contains no uronic acid. While variations in amino sugar ratios are present, they are not large, and they do not conform to the well-known pattern of age changes found in mammalian systems. Whilst a falling off in the total amount of mucopolysaccharide with age is indeed found, much of this can be accounted for by changes in the degree of calcification as manifested by the results of the ash measurements.

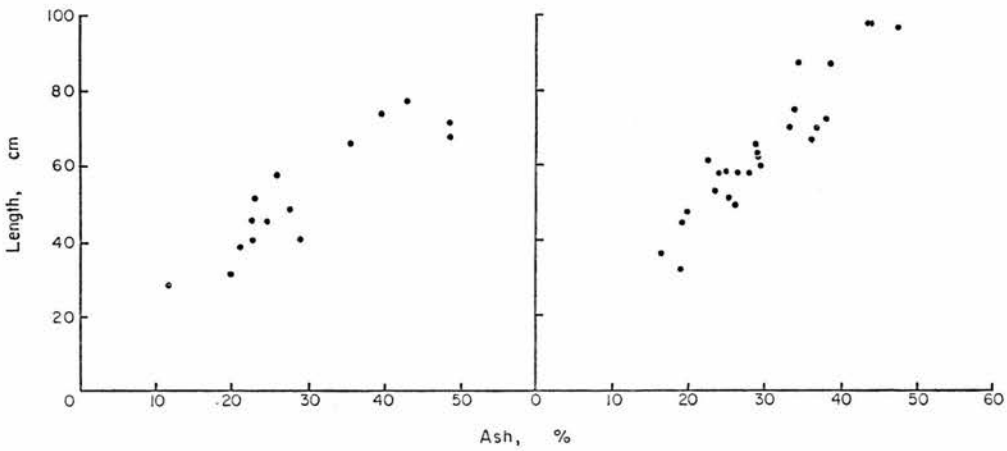


FIG. 1. Variation in ash content with length (as age parameter) in *Squalus acanthias* pectoral cartilage. Left, males; right, females.

*Inorganic changes*

Figure 1 shows the variation of ash content of dried cartilage plotted against length of the animal. Length is usually regarded as an age parameter in fish (rather than, say, weight). A difference is found between males and females in this respect. Holden & Meadows (1962) also noticed that males and females in this species had different lengths at the same age. The regression coefficient for males in the present study is 0·63, and for females, 0·44. Correlation coefficients are 0·90 and 0·93 respectively.

## DISCUSSION

Glucosamine-containing polysaccharides do not form a large part of the structural carbohydrate of *Squalus acanthias* cartilage. In human cartilage, Stidworthy *et al.* (1958) and Kaplan & Meyer (1959) showed that keratosulphate (measured as glucosamine) accounts for half the total mucopolysaccharide material in old age. In *Squalus acanthias*, only about 10 per cent of the total polysaccharides is keratosulphate. The significant point is that there is, from birth, no change with age in the ratio of the hexosamine-containing material. The tissue becomes calcified, as the ash results indicate, and does so to a surprising degree with advancing age, and the concentration of total polysaccharide does decrease, especially in the very old. Much of this change is, however, a consequence of the increasingly inorganic nature of the tissues. This is a real age change involving, probably, a shift from binding water to binding calcium. Ageing, in this context, suggests a shift from fluid viscoelastic resistance to compression forces to the more rigid bone-like behaviour (cf. Fessler, 1957). Apparently, the change to binding more calcium does not require a change in the mucopolysaccharide system. There is no evidence from this study for the mammalian-type changes occurring in elasmobranchs, involving a change from chondroitin-4-sulphate to chondroitin-6-sulphate with a simultaneous increase in keratosulphate (Seno *et al.*, 1965). One fraction of mammalian cartilage mucopeptide isolated by Seno *et al.* (1965) contained a doublet of keratosulphate and chondroitin-6-sulphate linked by a short peptide chain. The appearance of this sort of structure in mammalian cartilage could account for the chemical ageing changes observed. In shark cartilage (*Scoliodon walbechmi*) Furuhashi (1961) found that the infra-red spectrum is identical with that of chondroitin sulphate-C (chondroitin-6-sulphate), but is more highly sulphated than mammalian chondroitin sulphates. Anderson & Meyer (1962) also report that shark cartilage (species unknown) consists only of chondroitin-6-sulphate and keratosulphate. Seno *et al.* (1965) describe two main types of cartilage keratosulphate in mammals, distinguishable by their difference in behaviour towards alkali and pronase digestion. These workers associate keratosulphate II with chondroitin-6-sulphate in ageing cartilage, i.e. the pronase-resistant, alkali-sensitive form. It is not clear whether the keratosulphate from elasmobranch cartilage belongs to either of these groups, or whether the keratosulphate and chondroitin sulphate in elasmobranch cartilage are wholly or partially covalently linked. No figures are yet available for sialic acid in *Squalus* cartilage; in ox and horse nasal septum cartilage, Szirmai & Doyle

(unpublished) found changes in sialic acid concentration which paralleled those of keratosulphate. The differences in ageing behaviour between elasmobranch and mammalian cartilage can be explained by assuming that elasmobranch cartilage has already "aged" at birth; in the species reported in this paper it is noteworthy that the gestation period is long—some 2 years. Lash & Whitehouse (1960) in a study of (unnamed) shark cranial cartilage recorded analytical differences between embryo cartilage and adult cartilage, which they attributed to changes in the relative proportions of chondroitin sulphate and keratosulphate. Their results, however, do indicate a marked *rise* in uronic acid with age, a situation not observed elsewhere.

There is no obvious reason why elasmobranchs should "age" in any aspect more quickly than mammals. Indeed, since fish spend their lives at lower and more equable temperatures than most mammals, the reverse might be thought reasonable. A lowered ambient temperature environment is known to increase both the growth and life-span within certain fish species (Liu & Walford, 1966). In rat costal cartilage, Anderson & Odell (1960) consider that the changes in mucopolysaccharide ratios represent a maturation rather than a senescence. X-radiation, which hastens the appearance of ageing changes in mammals, has, apparently, no effect on the ageing change in mammalian cartilage mucopolysaccharides.

## SUMMARY

The proportions of the main mucopolysaccharides in pectoral cartilage from *Squalus acanthias* remain constant with age. The mineral content varies linearly with age and, at maximum, accounts for nearly half the dry weight.

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